2009

ABSTRACT BOOK

NCI TRANSLATES

NCI Translational Science Meeting

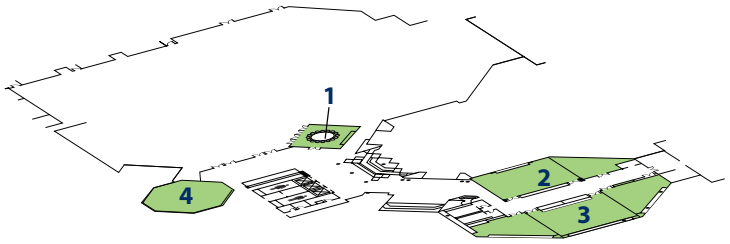
November 5–7, 2009 • Sheraton Premiere Hotel • Vienna, VA

Sheraton Premiere at Tysons Corner

8661 Leesburg Pike • Vienna, Virginia 22182 • United States
Phone (703) 448–1234

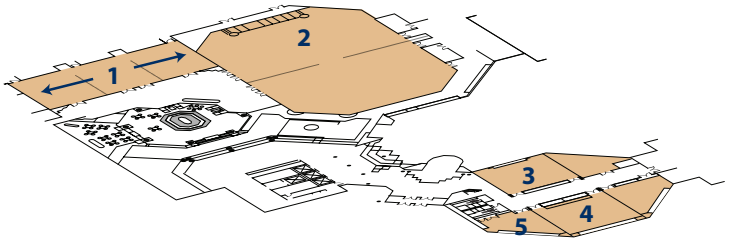
Mezzanine Level

1) Daniel M. Ross Boardroom
2) Mezzanine 2
3) Mezzanine 3
4) Capital Club



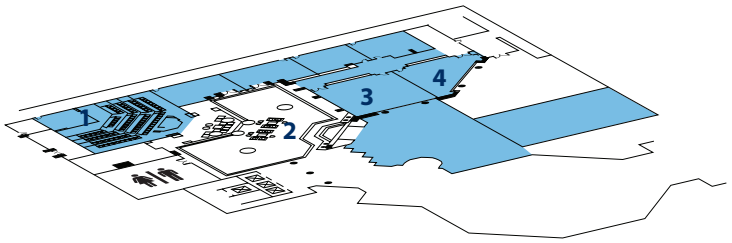
Lobby Level

1) Junior Ballroom
2) Grand Ballroom
3) Pavilion 22
4) Pavilion 23
5) Pavilion 25



Conference Level

1) Conference Theater
2) Exhibit Hall Foyer
3) Conference Room 6
4) Conference Room 8



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20892

Welcome to NCI's second annual Translational Science Meeting, an event that will showcase the exciting research of your fellow investigators. We hope that this gathering provides you a convenient venue in which to forge new collaborative opportunities with colleagues who are pursuing mutually beneficial science. Meeting participants represent all the key areas that are critical to advancing fundamental science to clinical application. In addition, this unique conference will provide opportunities in translational research for accelerated development and ultimate use in the clinical care of patients.

In 2007, the Translational Research Working Group (TRWG) defined a series of key pathways to move laboratory discoveries into early-stage clinical trials. The beacon of molecular oncology's promise was well recognized; however, the TRWG understood that to realize the full potential of these molecular discoveries would require enhanced coordination and collaboration. The ultimate aim is to accelerate the efficient development of the most promising opportunities in translational research.

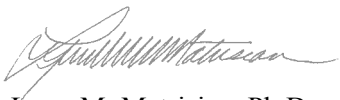
Since our last meeting, we have been hard at work implementing the TRWG's recommendations that NCI create a transparent process to gather the best ideas from the community and prioritize them for accelerated development. As a pilot of the pathway approach, a Request for Information (RFI) was released on the Immune Response Modifiers pathway, and the information received from the scientific community is under careful consideration by the Institute. A similar approach is planned for the Interventive Devices, Lifestyle Alterations, Biospecimens Assessment Modalities, and Imaging Assessment Modalities pathways. To address the Agents and Biologics pathway, we have developed a joint collaboration between NCI's Experimental Therapeutics program (NExT) and the Coordinating Center for Clinical Trials' Translational Research Support Team (TRST), to streamline drug development for the cancer community.

We all want more effective assessments, interventions, and therapies that will benefit cancer patients, and this meeting plays a critical role in advancing our collective commitment to progress in translational research for human benefit. I have no doubt that the poster sessions and ensuing discussions will expand collaboration and synergy among participants and NCI-supported investigators, ultimately aiding patients, clinicians, and scientists around the country.

Let us share the outstanding science of our colleagues so that we can maximize opportunities to accelerate translation to serve our patients and the public. Our success relies on the always-generous contribution of your time and engaged participation. For that, we, along with all of our NCI colleagues, are most appreciative.

John E. Niederhuber, M.D.
Director
National Cancer Institute

Sheila A. Prindiville, M.D., M.P.H.
Director
Coordinating Center for Clinical Trials



Lynn M. Matrisian, Ph.D.
Special Assistant
National Cancer Institute

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NCI Translational Science Meeting Abstracts
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Presenting authors' names appear in bold, underlined text.
(Note: Changes may have been made after the abstract book was printed.)

1 Negative Selection of Circulating Tumor Cells That Are Cancer Stem Cell-Like From Peripheral Blood of Cancer Patients

Jeffrey J. Chalmers[1], James C. Lang[1], Kris R. Jatana[1], David Schuller[1], Amit Agrawal[1], **Maciej Zborowski**[2]
[1]The Ohio State University, Columbus; [2]Cleveland Clinic, Cleveland, OH

The last decade brought an increased interest in studying the significance of the presence of circulating tumor cells (CTCs) in the peripheral blood and disseminated tumor cells (DTCs) in bone marrow of cancer patients. The rare CTC and DTC detection is based on immunophenotyping and PCR analysis and, more recently, aided by magnetic cell separation. The presence of CTC and DTC by positive PCR results has been reported for different types of cancer, particularly breast and melanoma cancers (Patel et al. 2008; Cristofanilli et al. 2004; Muller et al. 2005; Mocellin et al. 2006). The current methods of CTC and DTC isolation by magnetic separation relies on a positive selection approach to separate and identify the CTC and DTC based on the surface expression of an epithelial marker (such as EpCAM). We have recently shown successful isolation of CTCs from peripheral blood by negative selection approach that does not require prior knowledge of defined cancer markers (Yang L. et al. 2009; Balasubramanian et al. 2009). Only normal blood cells (erythrocytes and leukocytes) are targeted for removal, thereby allowing the putative CTCs to be recovered in system effluent for further analysis. In this manner we avoid an unintentional biasing for the type of CTCs isolates from the blood (such as for EpCAM-positive CTCs only) that has been recently identified as a limitation of the positive magnetic cell separation technology (Sieuwerts et al. 2009). The study is open for patients with squamous cell carcinoma of the head and neck and breast cancer patients at the Ohio State University site (under IRB-approved protocols). Patient accrual is ongoing, and we have collected and analyzed samples from 40 breast cancer patients and over 60 head and neck cancer patients. The preliminary data show not only presence of CTCs in the blood of those patients, but also identify a subpopulation of CTCs with mesenchymal immunophenotype that is characteristic of cancer stem cells (by confocal imaging). Concurrently, the negative magnetic selection technology is being commercialized to make it available to other clinical sites.

(Supported by R01 CA62349, R01 CA97391 and R33 CA81662.)

2 Understanding and Targeting Cancer Stem Cells

Curt Civin[1,2], Saul Sharkis[2], Robert Brodsky[2], Richard Jones[2], Donald Small[2], Alan Friedman[2], Steven Goodman[2], Mark Levis[2], William Matsui[2], Sarah Wheelan[2]
[1]Greenebaum Cancer Center, University of Maryland School of Medicine; [2]Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine

As we identify and isolate normal tissue-specific stem cells and Leukemia Stem Cells (LSCs), we are assessing the activity of and role of critical molecular signaling pathways in normal stem-progenitor cells and LSCs. For example, our Program Project Grant studies include the following directions: (1) Separate LSCs using properties that distinguish normal tissue-specific stem cells from their differentiated progeny. In addition, we will investigate whether the divergent outcomes between “pediatric-type” and “adult-type” acute lymphoblastic leukemia (ALL) are the result of different stem cell populations. In clinical trials, we will test whether targeting ALL stem cells via inhibition of the hedgehog pathway (e.g., Smothened) or telomerase will improve the outcome in adult ALL. (2) Determine whether the FLT3 mutation is expressed in LSCs from patients with FLT3 mutant acute myeloid leukemias (AMLs). In addition, we will evaluate whether LSCs from patients with FLT3 mutant AML are heterogeneous with respect to response to in vivo treatment with FLT3 inhibitors. We will also study how other oncogenes (known to occur in associatation with mutated FLT3 in human leukemia cases) cooperate with mutant FLT3 to transform normal hematopoietic stem-progenitor cells into LSCs. The understanding gained may influence design of future clinical trials employing FLT3 inhibitors. (3) Quantify microRNA expression at defined steps of normal human and mouse hematopoietic development and in LSCs versus the bulk populations of leukemia cells in a given patient sample. In addition, we will determine the cell and molecular mechanisms by which selected microRNAs affect the differentiation and biology of primary human hematopoietic stem-progenitor cells and LSCs. This understanding may inspire clinical trials of microRNA mimics, microRNA antagonists, or drugs directed at pathways regulated by microRNAs.

3 The Pan-HDAC Inhibitor Vorinostat Interacts Synergistically With the Second-Generation Proteasome Inhibitor Carfilzomib in Human DLBCL Cells In Vitro and In Vivo

Girija Dasmahapatra, Beata Holkova, Dmitri Lembersky, Paul Dent,Richard Fisher, Jonathan Friedberg, **Steven Grant**

Massey Cancer Center, Virginia Commonwealth University Health Sciences Center; the James Wilmot Cancer Center, University of Rochester

Previous studies have shown a high degree of synergism between histone deacetylase inhibitors (HDACIs) and proteasome inhibitors (PIs) in human leukemia, multiple myeloma, and diffuse lymphocytic B-cell lymphoma (DLBCL) cells. Carfilzomib (C) is a second-generation, irreversible epoxyketone PI with preclinical activity against bortezomib-resistant tumors that is undergoing evaluation in multiple myeloma and other hematopoietic malignancies. Vorinostat (V) is a pan-HDACI that has been approved in CTCL and interacts synergistically with bortezomib. Our goal was to determine whether V might potentiate C lethality in DLBCL cells sensitive or resistant to bortezomib. Co-administration (24 hour) of marginally toxic concentrations of C (3–5 nM) with minimally toxic concentrations of V (1.0–1.5 μM) markedly increased cytochrome c release, caspase activation, and apoptosis in both GC (SU-DHL-4 and -6) and ABC (OCI-Ly10 and -19 DLBCL) cells. These events were accompanied by a marked increase in activation of the stress-related kinases SEK1 and JNK, AKT downregulation, and ERK1/2 activation. C abrogated V-mediated NF-κB activation in both ABC and GC DLBCL cells. DLBCL cells (both GC and ABC) stably transfected with JNK shRNA displayed significant protection from the C/V regimen, arguing for a functional contribution of JNK activation in C/V synergism. Cell cycle analysis of DLBCL cells exposed to C/V revealed a marked G2M accumulation. DLBCL (e.g., SUDHL-16) cells resistant to 10 nM bortezomib displayed only modest cross-resistance to C administered alone. Significantly, C and V interacted synergistically to induce apoptosis in bortezomib-resistant DLBCL cells. The C/V regimen induced pronounced apoptosis in primary samples obtained from three patients with DLBCL but exerted minimal toxicity in normal hematopoietic cells (i.e., CD34+ bone marrow cells). Finally, co-administration of C/V to nude mice inoculated with either GC or ABC DLBCL cells at doses that exerted relatively modest effects alone induced a marked inhibition of tumor growth in a xenograft DLBCL flank model. These findings indicate that C interacts synergistically with V in both ABC and GC DLBCL, including cells resistant to bortezomib, through a JNK-dependent process. They also demonstrate that C/V is active in primary DLBCL cells and is effective in vivo. Collectively, these data support further efforts to explore a therapeutic strategy combining C with V in patients with refractory DLBCL.

4 Inhibition of Breast Cancer Stem Cells (CSCs) by Novel STAT3 Inhibitors

Bhuvanesh Dave, David J. Tweardy, C. Kent Osborne, Melissa Landis, Lacey Dobrolecki, **Michael T. Lewis**, Jenny C. Chang

[1]Lester and Sue Smith Breast Center, Baylor College of Medicine, Houston, TX; [2]Section of Infectious Diseases, College of Medicine, Houston, TX

Background: Many patients relapse over time despite initial response to systemic therapy. One explanation is that a rare subpopulation of CSCs with tumorigenic potential is intrinsically resistant to therapy. Previously, we had shown clinically in human breast cancer patients that residual tumors after chemotherapy (1) are enriched for the tumorigenic CD44+/CD24-/low population, (2) show enhanced mammosphere-forming efficiency (MSFE), and (3) display an increase in outgrowths in xenograft transplants in immunocompromised SCID/beige mice. We had identified a cancer stem cell signature of CD44+/CD24-/low mammosphere-forming cells derived from human breast cancer biopsies. The top canonical pathway in CSC self-renewal identified included STAT3 signaling. We hypothesized that inhibition of STAT3 will lead to a decrease in the number of breast CSCs. We had developed two molecules, a G-quartet oligodeoxynucleotide (GQ ODN) and a small molecule (Cpd 188), that selectively inhibited Stat3 activation through the Stat3 Src homology (SH) 2. Furthermore, we had performed 2-dimensional similarity screening using the scaffold of Cpd188 as the query structure and derived 39 second-generation compounds that inhibited Stat3 binding to its phosphopeptide ligand by surface plasmon resonance (SPR). **Methods:** From biopsies of primary human breast cancers, we have established primary xenografts in immuno-compromised SCID beige mice, which were genotypically identical to the primary breast cancers. For this pilot study, a triple negative (ER-negative, PgR-negative, and HER-2) tumor line (n=3 mice/group) was treated with two doses of vehicle, the small molecule Cmpd188 (125mcg and 250mcg), GQ-ODN (125mcg and 250mcg), and a control non-specific ODN (GQ-II) (125 or 250 mcg). The animals were sacrificed after only 2 days of treatment and MSFE determined. In addition, we tested the 39 second-generation compounds for the ability to decrease MSFE. **Results:** In these short-term experiments, a statistically significant reduction ($p<0.05$) was observed in the MFSE with Cmpd 188250 mcg) and GQ_ODN (125 and 250 mcg) versus control. Of the panel of 39 second generation STAT3 inhibitors, 11 derivatives significantly decreased MSFE in two triple-negative cell lines (SUM159PT and HS578T). **Conclusions:** STAT3 inhibition significantly reduced CSC both in vivo and in vitro. Newer generation inhibitors with increased efficacy will be tested and thus may improve existing cancer therapies.

5 The Global Profiles of Specific Histone Modifications Measured by High-Throughput Reverse-Phase Protein Microarrays Provide New Insights Into Cancer Biology

Christopher B. Devor, Russell W. Bandle, Alexandra E. Kovach, John Janik, Barry L. Gause, Louis M. Staudt, Wyndham H. Wilson, Stefania Pittaluga, Elaine S. Jaffe, Katherine R. Calvo, Hye-Jung Chung, **David Levens**

National Cancer Institute, National Institutes of Health, Bethesda, MD

The global profiles of specific histone modifications measured by high-throughput reverse-phase protein microarrays provide new insights into cancer biology. Chromatin immunoprecipitation has led to a proliferation of studies revealing close relationships between gene expression and the post-translational acetylations, methylations, and phosphorylations of histones. Here we used a novel, high-throughput approach to measure the global extent of these residue-specific modifications across many samples, including human cancer tissues and cell lines. Comparisons between these sample populations provide insight into the regulation of chromatin dynamics in physiological processes and the pathological processes operating in disease. We tested the compatibility of over 80 chromatin antibodies on our reverse-phase protein lysate array (RPA) platform. From these antibodies, we established RPA immunostains specific for more than 30 unique covalent histone modifications. For each of our working antibodies, we stained and analyzed an RPA with 120 unique lymphoma or hyperplasia tissue biopsy lysates. The biopsy samples were previously diagnosed as Follicular Hyperplasia (FH, n=21), Follicular Lymphoma Grade 1 (FLG1, n=16), Grade 2 (FLG2, n=14), Grade 3 (FLG3, n=9), Chronic Lymphocytic Leukemia (CLL, n=15), Mantle Cell Lymphoma (n=5), Diffuse Large B-Cell Lymphoma (DLBC, n=23), Burkitt's Lymphoma (n=5), and Anaplastic Large Cell Lymphoma (ALCL, n=8). The resulting RPA dataset associates specific patterns of histone modifications with various pathological entities. Furthermore, certain expression patterns correlate with cancer type and aggressiveness, suggesting that the global activity of these modifications may be useful for diagnostic, prognostic, and targeted molecular therapeutic purposes. Moreover, because RPA is a versatile, high-throughput platform to monitor concurrent and sequential changes in chromatin modification in response to physiological (e.g., serum-stimulation of fibroblasts) or pharmacological manipulations, it is well suited to evaluate the efficacy of histone targeting therapeutics in a clinical setting.

6 Translational Research Efforts at the Norris Cotton Cancer Center to Target Local and Distant Tumor Recurrence

James DiRenzo, Gary Schwartz , Kari Rosenkranz, Mark Israel

Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Dartmouth Medical School

The Norris Cotton Cancer Center (NCCC) aims to develop improved strategies to prevent and cure cancer through interdisciplinary research and translate these strategies into clinically applicable preventative and therapeutic approaches. Recently, the NCCC has developed a translational research platform geared towards population-based preventative interventions and clinically based therapeutic research. The translational research platform has a two-fold mission. It seeks to integrate the research of over 125 basic and preclinical scientists affiliated with six NCCC Research Programs. Additionally, it has established disease site-specific scientific directorships to identify specific clinical obstacles and develop research-based strategies to meet those challenges. At NCCC, there is specific translational focus on cancer recurrence, which is a major cause of cancer mortality. Recurrence is the result of a subset of cells in a tumor that are resistant to therapy and tumorigenic. Research focused upon the isolation and analysis of these cells in breast cancer models indicates that they utilize cellular quiescence to resist therapeutic intervention and preserve their long-term tumorigenicity. Analysis of regulatory mechanisms governing entry into and maintenance of cellular quiescence in this fraction indicates that Notch signaling promotes quiescence and that disruption of Notch signaling was sufficient to drive quiescent populations into a proliferative phase. These findings have led to the translational hypothesis that treatment of breast cancer patients with γ-secretase inhibitors in the setting of neoadjuvant or adjuvant chemotherapy may sensitize chemoresistant cells by forcing them into the cell cycle. Laboratory endpoints will detect the tumorigenic population within primary breast tumors and ascertain its proliferative status. These endpoints will be deployed in samples derived from a Phase 1b clinical trial of a γ-secretase inhibitor in the setting of neoadjuvant treatment. We aim to determine whether γ-secretase inhibition causes a transition from quiescence proliferation among therapeutically resistant cells and to determine whether this leads to more efficient therapeutic outcome and reduced risk of breast cancer recurrence. Similar translational studies are underway to determine the applicability of this strategy to other solid tumors.

7 Development of Vorinostat in Metastatic Colorectal Cancer

Marwan Fakih[1], Lakshmi Pendyala[1], Merrill Egorin[2], Gerald Fetterly[1], Igor Espinoza-Delgado[3], James Zwiebel[3], Josephia Muindi[1], Robert Diasio[4]

[1]Roswell Park Cancer Institute; [2]University of Pittsburgh; [3]Cancer Therapy Evaluation Program, National Cancer Institute; [4]Mayo Clinic

Baseline or acquired thymidylate synthase (TS) overexpression is associated with 5-fluorouracil (5-FU) resistance in patients with colorectal cancer (CRC). Vorinostat, an FDA-approved histone deacetylase (HDAC) inhibitor, decreases TS expression via translational repression in CRC cell lines and xenografts, therefore enhancing 5-FU anti-tumor activity. We investigated escalating doses of vorinostat combined with a fixed dose of 5-FU, oxaliplatin, and leucovorin (FOLFOX) in 21 patients with refractory CRC. The maximum tolerated dose (MTD) of vorinostat was 300mg orally twice daily for 7 days every 2 weeks with FOLFOX being administered on days 4 and 5 of vorinostat. Dose-limiting toxicities (DLT), fatigu, and diarrhea, were noted at the 400mg orally twice-daily dose level. Vorinostat dose levels (DL) 300 and 400mg taken orally twice daily experienced increased grade ≥3 thromboyctopenia. Pharmacokinetic (PK) analysis at the MTD revealed suboptimal vorinostat PK for TS modulation: Cmax=1.4 ± 0.7 μM with T1/2 of 2±2.3 hours. No trends in TS modulation were noted by immunohistochemistry (IHC) or RT-PCR. Higher DL of vorinostat experienced decreased 5-FU clearance compared to lower DL. In order to optimize vorinostat concentrations (>2μM), we conducted a Phase I study of escalating doses of vorinostat every day for 3 days combined to a fixed dose of 5-FU/LV on days 2–3, repeated every 2 weeks. Twenty-seven patients with refractory solid tumors enrolled. Two DLTs (fatigue and hand and foot syndrome) were seen in two-thirds of patients at DL 2000mg. Vorinostat MTD was 1700mg every day for 3 days. Vorinostat Cmax at the MTD exceeded 6μM, and the C8hrs exceeded 1μM (2 patients analyzed). In an expanded MTD cohort, 5-FU PKs were performed with and without vorinostat without evidence of PK interaction. This was consistent with the lack of dihydropyrimidine dehydorgenase (DPD activity assay) modulation by vorinostat at the MTD. In two patients with pre- and post-vorinostat tumor biopsies at the MTD, no TS downregulation was noted by IHC (TS106), while RT-PCR data was uninterpretable due to low baseline expression. Efficacy was explored in patients with a diagnosis of metastatic CRC (24 patients). All CRC patients were 5-FU-, oxaliplatin-, irinotecan-, and cetuximab-refractory. Twelve of 24 patients had SD, and one patient had a PR (ongoing for more than 1 year). The median PFS and OS of this cohort were 4.4 and 9.2 months, respectively. These results compare favorable with historic chemorefractory CRC controls. Vorinostat plus 5-FU/LV development is ongoing in a Phase II study in refractory CRC.

8 Potentiation of Histone Deacetylase Inhibitor Activity by Agents That Interrupt the NF-κB Pathway: Translational Implications for Hematologic Malignancies

Steven Grant, Yun Dai, Roberto Rosato, Mohamed Rahmani, Paul Dent, Beata Holkova, John Roberts

Departments of Medicine and Biochemistry, Massey Cancer Center, and the Institute for Molecular Medicine, Virginia Commonwealth University Health Center

Histone deacetylase inhibitors (HDACIs) are epigenetic agents recently approved in cutaneous t-cell lymphoma and under investigation in hematopoietic malignancies. Previous studies have shown that HDACIs induce p65/RelA acetylation, thereby promoting NF-κB activation, leading to transcription of pro-survival NF-κB-dependent genes, including MnSOD2, Bcl-xL, and XIAP. Conversely, NF-κB inhibition opposes HDACI-mediated p65/RelA acetylation / activation, thereby downregulating anti-apoptotic proteins and triggering cell death via a JNK-dependent process (Dai et al., Mol Cell Biol 25:5429, 2005). HDACIs interact synergistically with certain cytotoxic agents (e.g., fludarabine) in malignant hemato-poietic cells through induction of oxidative damage, NF-κB inactivation, DNA repair protein downregulation, and DNA damage. We have now developed a strategy to enhance HDACI activity in hematopoietic malignancies by interrupting cytoprotective signaling pathways. In one approach, we exploited the ability of the CDK inhibitor flavopiridol to inhibit IKK and the cyclinT/CDK9 pTEFb transcription complex, blocking transcription of p21CIP1 and XIAP by HDACIs. This led to a multi-institutional Phase I trial of vorinostat and flavopiridol in refractory AML/MDS. A related strategy employs proteasome inhibitors (PIs; e.g., bortezomib) to enhance HDACI lethality. PIs prevent NF-κB activation by blocking IκBα degradation directly but may also amplify the lethal consequences of HDACI-induced aggresome dysfunction by disrupting protein degradation. Synergistic preclinical interactions between vorinostat and bortezomib have been observed in malignant lymphoid cells (e.g., MM, DLBCL), and recently bortezomib has been shown to interact synergistically with depsipeptide (romidepsin) in CLL cells in association with NF-κB inactivation (Dai et al., Clin Cancer Res 14:549, 2008). Collectively, these preclinical findings prompted the development of (1) a multi-institutional Phase II trial of vorinostat and bortezomib in refractory DLBCL and mantle cell lymphoma; (2) a Phase I trial of romidepsin and bortezomib in refractory CLL; and (3) a Phase I trial of the Class I HDACI belinostat (PXD-101) and bortezomib in refractory AML/MDS. In summary, these initiatives should allow us to test the hypothesis that preclinical evidence that NF-κB inactivation promotes HDACI lethality can be translated into improved HDACI activity in patients with diverse hematologic malignancies.

9 Inhibition of Tumorigenesis and Immune Suppression With Inhibitors of the STAT-3 Pathway

Amy B. Heimberger[1], Ling-Yuan Kong[1], Jun Wei[1], S. Farzana Hussain[1], Mohamed Abou-Ghazal[1], Arup Chakraborty[1], Izabela Fokt[1], Wei Qiao[1], Wei Sun[1], Gary E. Archer, Jr.[2], John H. Sampson[2], Gregory N. Fuller[1], Elizabeth A. Grimm[1], Howard Colman[1], Raymond Sawaya[1], Frederick F. Lang[1], Waldemar Priebe[1]

[1]The University of Texas M. D. Anderson Cancer Center; [2]Duke University Medical Center

The signal transducer and activator of transcription (STAT)-3 pathway is expressed across a wide variety of malignancies, including central nervous system (CNS) gliomas and melanoma. Phosphorylation of STAT-3 (p-STAT-3) drives the fundamental components of tumorigenesis and is a primary mediator of immune suppression. P-STAT-3 is induced in a variety of immune subsets upon encountering the tumor environment or the secreted products from the tumor. Specifically, p-STAT-3 induces FoxP3 in T regulatory cells (Tregs), downmodulates co-stimulatory molecules and pro-inflammatory cytokines in antigen-presenting cells, and induces immunosuppressive CNS microglia/macrophages. Cancer stem cells (CSCs) isolated from human CNS gliomas, which recapitulate many of the features of human gliomas and are mediators of chemo- and radiation resistance, produce a variety of immune suppressive products such as macrophage inhibitory cytokine 1 (MIC-1) and express CTLA-4 and B7H1 that trigger T cell apoptosis, inhibit T cell proliferation and function, and induce Tregs. The STAT-3 pathway is overactive in CSCs, and treatment with either p-STAT-3 inhibitors, STAT-3 siRNA, or differentiation reverses the CSC induction of T cell apoptosis and Tregs and restores T cell effector functions. Furthermore, the p-STAT-3 inhibitors reverse the immune suppressive phenotype of human microglia by reducing MIC-1 production. Thus, STAT-3 inhibitors could be utilized to modulate the redundant immunosuppressive mechanisms in cancer patients and could be used as chemotherapy modulators or radiation sensitizers. We have developed orally administered small molecule inhibitors (WP1066, WP1193) of the p-STAT-3 pathway with excellent CNS penetration and a favorable toxicity profile. Long-term survival was observed in 80% of mice with established intracerebral syngeneic melanoma (B16) treated with WP1066 ($p<0.05$) and also markedly enhanced the in vivo efficacy of IFN-alpha. Although WP1066 did not induce immunological memory or enhance humoral responses, it reduced the production of immunosuppressive cytokines and chemokines, inhibited Tregs, and increased the cytotoxic immune responses of T cells. This in vivo therapeutic efficacy is ablated in nude model systems and with in vivo depletions of CD8+ effector T cells. Thus, the p-STAT-3 inhibitors may have utility in combination with other immunotherapy and vaccine approaches. Our intention is to bring the p-STAT-3 inhibitors into Phase I/II clinical trials within the next 18 months for patients with CNS melanoma metastasis who are typically excluded from clinical trials and in patients with primary gliomas.

10 Phase I Trial of Carfilzomib (PR-171) in Combination With Vorinostat (SAHA) in Patients With Relapsed/Refractory B-Cell Lymphomas

Beata Holkova[1], Girija Dasmahapatra[2], John Roberts[3], Richard Fisher[4], Jonathan Friedberg[5], Steven Grant[6]

[1]Virginia Commonwealth University/Massey Cancer Center; [2]James Wilmot Cancer Center, University of Rochester

Despite high initial response rates, relapse/refractory B-cell lymphomas are common and associated with poor outcomes. New treatment strategies are urgently needed. Bortezomib, a dipeptidyl boronic acid that is a reversible proteasome inhibitor (PI), is FDA-approved for the treatment of mantle cell lymphoma. Unfortunately, both primary bortezomib resistance and recurrence with refractory disease are common. Carfilzomib, an epoxyketone, is a novel, irreversible PI that has shown preclinical activity in malignant lymphoid cells resistant to bortezomib. Vorinostat, a prototypical pan-histone deacetylase inhibitor (HDI), is FDA-approved for the treatment of refractory cutaneous T cell lymphoma and is being evaluated as a single agent and in combination regimens in relapse/refractory B-cell lymphomas. Recently, a large number of HDI/PI trials, generally involving various HDIs in conjunction with bortezomib, have been launched in both hematological and non-hematologic malignancies. Several considerations make carfilzomib an attractive candidate for combination regimens involving HDIs: (1) diminished neurotoxicity, (2) irreversible proteasome inhibition, (3) tolerability of a more chronic administration schedule compared to bortezomib, and (4) preclinical and clinical evidence of activity in the bortezomib-resistant setting. Recent preclinical studies indicate that carfilzomib and vorinostat interact synergistically to induce apoptosis in various NHL cell types in association with NF-kB inactivation and induction of the JNK stress-related pathway. Notably, such interactions have been documented in highly bortezomib-resistant DLBCL cells, primary human DLBCL cells, and xenograft models of DLBCL. To test the hypothesis that these preclinical findings can be translated into improved outcomes for patients with relapsed/refractory B-cell lymphomas, a multi-institutional Lymphoma SPORE Phase I trial will shortly be initiated. The specific aims of this trial are to (1) determine the maximum tolerated dose for the combination of vorinostat and PR-171 and characterize the safety and the toxicities of the combination; (2) demonstrate adequate techniques for the assessment of pharmacodynamic responses of primary lymphoma cells to this regimen with respect to effects on activation of NF-kB (nuclear RelA), activation of JNK, and expression of the pro-apoptotic protein Bim; and (3) document pharmacodynamic responses observed in the course of the dose-finding study. This clinical trial may serve as a prototype for translating promising preclinical findings developing within a Lymphoma SPORE directly into the clinical arena in the form of a hypothesis-based Phase I study.

11 DNA Methylation and Chromatin Modifications: Mechanisms and Applications in Cancer Therapy

Samson T. Jacob, John Byrd, Christoph Plass, Mark Parthun, Said Sif, David Jarjoura, Tasneem Motiwala, Kalpana Ghoshal, David Lucas, Michael Grever

Departments of Molecular and Cellular Biochemistry, and Internal Medicine, and the Comprehensive Cancer Center, The Ohio State University, Columbus

This translational research program consists of five projects including a Phase I clinical project. One of the goals is to identify differentially methylated genes in chronic lymphocytic leukemia (CLL) relative to B-cells that can potentially predict prognosis and outcome of CLL. We previously showed that the death-associated protein kinase gene (DAPK) is silenced in CLL by promoter methylation. We are now elucidating the mechanisms by which this gene is silenced in a murine model of human CLL (TCL1 transgenic mice). Another project focuses on identification of CLL patients based on methylation status of the novel tumor suppressor gene encoding receptor-type protein tyrosine phosphatase PTPROt and identification of PTPROt-mediated signaling pathways regulating apoptosis. We have further demonstrated PTPROt suppression in TCL1 murine model of CLL at early stage of CLL development. A third project is to characterize the full spectrum of core histone variants and their post-translational modifications in primary CLL relative to normal B cells, as well as to characterize changes in these modifications induced by histone deacetylase (HDAC) inhibitors. This study showed a relatively large proportion of two-histone H2A variants in primary CLL cells compared to B cells. We are correlating changes in the levels of these histone variants with other CLL biomarkers and clinical outcomes and exploring their potential role in the silencing of specific tumor suppressors, PTPROt and ID4. A fourth project is concerned with elucidation of the role of chromatin remodeling factors—specifically BRG1 and hBRM and their associated proteins—in the development of CLL. A recent study showed that the arginine histone methyltransferase PRMT5 interacts with these chromatin remodelers and causes transcriptional silencing. The current study includes identification of components of PRMT5-containing complex involved in the regulation of expression of key target genes that include PTPROt, DAPK, and ID4. The final project is to perform preclinical work with novel HDAC and DNMT inhibitors and extend to Phase 1 trials specifically with OSU-HDAC-42 (an HDAC inhibitor developed at Ohio State University) in CLL, particularly in relapsed/refractory CLL. Since the DNA hypomethylating agent Decitabine was not effective in CLL, we are developing alternative mechanisms or other agents (e.g., miRs targeting DNMTs, novel non-nucleoside DNA hypomethylating agents) with the goal of re-activating silenced genes such as DAPK, PTPROt, and ID4.

12 Expression of Putative Stem Cell Markers Is Heterogeneous in Pancreatic Tumors and Direct Xenografts Derived From These Tumors

Michael P. Kim, Woongyoung Choi, Jason B. Fleming, Huamin Wang, Thiru Arumugam, Craig D. Logsdon, David J. McConkey, James L. Abbruzzese, **Gary E. Gallick**

The University of Texas M. D. Anderson Cancer Center

One of the most critical problems in pancreatic cancer treatment is drug resistance, and little success has been achieved in the design of therapeutic strategies to overcome this problem. In some tumor cells, such as putative cancer stem cells (CSCs), drug resistance may be inherent. Yet, the biological properties and abundance of CSCs in pancreatic tumors remain poorly understood. We have examined three markers considered important in CSCs: CD133, ALDH, and c-Met. Analyses of tissue microarrays (TMAs) demonstrate substantial heterogeneity in expression of these markers. To determine which of these cell surface proteins might correlate best with cells that manifest stem cell-like biology, we developed a direct xenograft program from untreated and previously treated patients. Tumors derived directly from patients show more glandular morphology and have a much more abundant stromal component than is observed in tumors resulting from orthotopic implantation of "well-established" pancreatic cell lines. Further, cell lines derived from the direct xenografts show similar properties, suggesting that these models retain the complex genetic and epigenetic alterations present in the original patient tumors and thereby recapitulate the behavior of pancreatic cancer in patients. In accord with results from TMAs, wide variation in expression of the above markers are observed, ranging from less than 3% to greater than 40%. To determine which markers best correlate with CSCs, FACS sorting was used, and limiting dilutions of ALDH+/CD133+, ALDH+CD133-, ALDH-/CD133+, and ALDH-/CD133- cells were implanted into NOD-SCID mice. In a patient tumor where less than 3% of cells expressed ALDH, tumors formed in NOD-SCID mice from implantation of 1000 ALDH+ cells, irrespective of the status of CD133, suggesting that in this patient xenograft ALDH is the critical marker of CSCs. Similar experiments from direct xenografts expressing high ALDH and low CD133 are in progress. From these experiments we will be able to assess the phenotypic diversity of pancreatic tumor CSCs and whether large differences in the abundance of a CSC marker such as ALDH are prognostic. Also, we are determining the relationship of expression of these markers to that of c-Met, itself implicated as a CSC marker and for which small molecule inhibitors are being tested in clinical trials, the results of which may guide future therapeutic strategies.

13 Population Pharmacokinetics of Vorinostat in Cancer Patients

Jill M. Kolesar[1], Richard C. Brundage[2], Jen Eickhoff, Joel M. Reid[3], Mathew M. Ames[3], Renee M. McGovern[3], William Schelman[1], Dona Alberti[1], Howard Bailey[1], George Wilding[1]

[1]University of Wisconsin Paul P. Carbone Comprehensive Cancer Center, Madison; [2]University of Minnesota, Minneapolis; [3]Mayo Clinic, Rochester, MN

Background: Vorinostat (suberoylanilide hydroxamic acid, SAHA) is an oral histone deacetylase (HDAC) inhibitor. The present study was conducted to describe the population pharmacokinetics (PK) of vorinostat and to assess the relationship between vorinostat disposition, patient covariates, and biomarkers. **Methods:** Fifty-one patients with advanced solid tumors from a Phase I study were included in the population PK model-building. Patients received vorinostat given orally either once daily (Step A) or twice daily (Step B) on days 1–14, combined with bortezomib IV on days 1, 4, 8, and 11 of a 21-day cycle. Plasma samples were collected over 8 hours after vorinostat administration on C1D1 (vorinostat alone, first dose), C1D2 (vorinostat and bortezomib), and C1D12 (vorinostat at steady state). The plasma concentrations of vorinostat and the acid and glucuronide metabolite were measured using a validated LC/MS/MS method, and noncompartmental methods were used to summarize pharmacokinetic parameters. Data will be analyzed by a nonlinear mixed-effects modeling approach using the NONMEM system Version VI. Xpose 4.0, and S-PLUS will be used for goodness-of-fit assessment and model evaluation to obtain total clearance (CL), intercompartmental clearance, and volumes of distribution for central and peripheral compartments. Stepwise covariate model-building will evaluate weight, body surface area, gender, performance status, age, UGT genotypes, HPS70 and p21 gene expression, toxicity (grade III-IV myelosuppression, gastrointestinal toxicity), concurrent chemotherapy (bortezomib), response, and number of cycles administered. Normal renal and hepatic function were required for study entry and not included as covariates. **Results:** Vorinostat PK results are available for the first 18 patients, all from Step A. Vorinostat accumulated with chronic dosing, with an AUC on day 1 of 1049 (hr*ng/mL), compared to 1412 (hr*ng/mL) by Day 12 ($p=0.022$). The acid metabolite also accumulated, with an AUC on day 1 of 8552 (hr*ng/mL), compared to 11096 (hr*ng/mL) by Day 12 ($p<0.0011$). **Conclusion:** From this preliminary analysis, both vorinostat and its acid metabolite accumulated with repeated dosing. A population PK model assessing the role of plasma concentration, response, toxicity, and downstream markers will be presented at the meeting.

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14 Discovery and Validation of Molecular Markers for Bladder Cancer

Cynthia LeBron, David Sidransky, **Mohammad O. Hoque**

Johns Hopkins College of Medicine, Baltimore, MD

Molecular markers for bladder cancer remain in the preclinical stages. To date, few advances have made it into the clinic. At present there are many diagnostic markers and diagnostic techniques that show promise. Indeed technologies such as expression arrays, proteomic approaches, molecular imaging techniques, RT-PCR, quantitative DNA PCR, and quantitative methylation-specific PCR all offer hope in detecting molecular changes in bladder cancer. It is now well accepted that cancer results from multiple accumulated, progressively transforming molecular alterations in clonal population cells. Early molecular changes offer the ability to molecularly diagnose, treat, and follow superficial lesions before the patient develops cancer. Additional molecular marker identification will aid in detection of this heterogeneous cancer with high sensitivity and specificity. The link between tobacco smoke exposure and bladder cancer has been identified to be the major risk factor for this disease, although the sequence of molecular events by tobacco smoke are not well understood. Therefore, to elucidate the molecular events for the progression of bladder cancer, we developed an in vitro cellular model for smoking-induced bladder cancer by exposing non-malignant human bladder epithelial cells to cigarette smoke. Morphological alterations and increased cell proliferation of non-malignant bladder cells were observed after 4 months of treatment. Soft agar colonies were observed after 6 months of treatment with cigarette smoke (CSE). CSE treatment also led to an increase in the migratory potential and invasive potential of cells, as well as a more spindle-like appearance. To understand the related molecular alterations associated with CSE, we analyzed a panel of genes that are reported to be altered in smoking-related cancers like lung, head and neck, esophageal, and bladder. This panel of genes included PTEN, p53, OLC1, AKT, p16, and RUNX3. PTEN is downregulated in the transformed cells at the translational level, while AKT and RUNX3 upregulated. We collected and stored frozen stocks of the CSE-treated cells with paired controls at each month, and extensive molecular studies are underway to identify molecular alterations that are related to initiation and progression of bladder cancer. Our study will facilitate the identification of molecular markers that are related to initiation of transformation, and addition of these markers with our previously identified molecular markers will allow development of non-invasive assay for the detection of bladder cancer using urine.

15 Perifosine, as a Single Agent or in Combination With Chemotherapy, Inhibits Neuroblastoma Tumor Cell Growth in In Vitro and In Vivo Pre-Clinical Models

Zhijie Li, Fei Tan, Jenna Tong, Amy McKee, **Carol J. Thiele**

Cell and Molecular Biology Section, Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD

Activation of Akt correlates with decreased event-free and overall survival in patients with neuroblastoma. Perifosine is a novel agent that modulates key signal transduction pathways, most notably Akt. The aim of this study was to determine the effectiveness of perifosine as an anti-cancer agent using neuroblastoma cells. We treated four human neuroblastoma cell lines (AS, BE2, KCNR, and NGP) with perifosine and evaluated cell proliferation using an MTS assay and apoptosis by evaluating caspase 3/7 levels. Immunodeficient mice were injected subcutaneously or in an orthotopic periadrenal location with neuroblastoma cell lines. After formation of palpable tumors, mice were randomized to treatment with perifosine or vehicle control and monitored for tumor progression. All four neuroblastoma cell lines treated with perifosine showed a decrease in cell proliferation in a concentration-dependent manner. These decreases coincided with inhibition of Akt phosphorylation and induction of caspase-mediated apoptosis. In in vivo studies, perifosine treatment led to delayed tumor growth in all four lines tested. This included complete abrogation of tumor formation in BE2 cells and marked regression of large tumors in AS cells. Activated Akt levels were markedly decreased in the tumors of perifosine-treated mice. In the least sensitive neuroblastoma cell line, NGP, combination of a sub-therapeutic dose of perifosine with an IC20 or IC50 dose of etoposide showed synergy and tumor regression. Perifosine inhibits activated Akt both in vitro and in vivo. In addition, it has potent anti-tumor effects in murine xenograft models alone or in combination with etoposide. Our study supports the continued investigation of perifosine as a therapeutic agent in patients with neuroblastoma.

16 Recombinant Human Erythropoietin Antagonizes Trastuzumab Treatment of Breast Cancer via Janus Kinase 2-Mediated Activation of Src and Inactivation of PTEN

Ke Liang[1], Francisco Esteva[2], Constance Albarracin[3], Yang Lu[1], Yong Li[3], Xinqun Li[1], Chun-Te Chen[4], Ching-Yi Yang[2], Giampaolo Bianchini[2], Gordon B. Mills[5], Gabriel N. Hortobagyi[2], John Mendelsohn[1], Mien-Chie Hung[4], **Zhen Fan**[1,5]

Departments of [1]Experimental Therapeutics, [2]Breast Medical Oncology, [3]Pathology, [4]Molecular and Cellular Oncology, and [5]Systems Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX

Recombinant human erythropoietin (rHuEPO) has been approved for preventing or alleviating cancer- and cancer-treatment-related anemia and fatigue since early 1990. Once thought to be only a hematopoietic cytokine, EPO is now known to be a pleiotropic cytokine characterized by remarkable cytoprotective activities in a variety of nonhematopoietic tissues, including neuronal, cardiac, retinal, and renal tissues, and more importantly in various cancerous tissues. The nonhematopoietic functions of EPO are biologically related to expression of EPO receptor (EpoR) recently discovered in these tissues. Because EPO can activate a cascade of cell signaling via EpoR-associated Janus kinase-2 (Jak2) that largely overlaps with the signaling pathways activated by human epidermal growth factor receptor-2 (HER2), we hypothesized that concurrent rHuEPO treatment might play a role in conferring resistance to trastuzumab, an anti-HER2 antibody used to treat HER2-positive breast cancer patients. In a series of 55 cases of breast cancer specimens, we found that 13 out of 15 HER2-positive cases had various degrees of positive staining for EpoR. We demonstrated that concurrent treatment of HER2/EpoR dual-positive breast cancer cells with trastuzumab and rHuEPO reduced the response to trastuzumab both in culture and in a nude mice xenograft model. We also discovered the underlying mechanisms involving Jak2-mediated activation of Src and inactivation of PTEN through which rHuEPO compensates trastuzumab-induced inhibition of cell signaling. Furthermore, we identified 1,941 women with breast cancer treated with rHuEPO at M. D. Anderson Cancer Center from December 1998 to February 2006; among those patients, 273 had received trastuzumab. We matched 50 metastatic breast cancer patients treated with first-line trastuzumab plus a taxane (with or without carboplatin) with another 50 metastatic breast cancer patients who received comparable treatments but no concomitant rHuEPO. We found only the concomitant administration of rHuEPO was significantly correlated with a lower likelihood of objective clinical response. The correlation between rHuEPO administration and reduced response to first-line trastuzumab was significant after adjustment for age, estrogen receptor and/or progesterone receptor status, grade, and carboplatin administration. Our results provide important preclinical and clinical evidence and mechanistic insights indicating that concurrent administration of rHuEPO and trastuzumab may be counteractive in patients with breast cancer that is positive for both EpoR and HER2.

17 Blocking Monoclonal Antibodies Directed Against CD47 Preferentially Enable Phagocytosis and Elimination of Human Acute Myeloid Leukemia Stem Cells

Ravindra Majeti, Mark P. Chao, Ash A. Alizadeh, Wendy W. Pang, Siddhartha Jaiswal, Irving L. Weissman

Stanford Cancer Center, Division of Hematology, and Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Palo Alto, CA

Human acute myeloid leukemia (AML) is organized as a cellular hierarchy that is initiated and maintained by rare self-renewing leukemia stem cells (LSC), which must be eliminated in order to cure the patient. We identified increased expression of CD47 on human AML LSC compared to their normal counterparts. CD47 is a cell surface molecule that serves as the ligand for SIRPα on the surface of phagocytes, which in turn transmits a dominant inhibitory signal for phagocytosis. In this way, CD47 essentially functions as a "don't eat me" signal. We hypothesized that increased CD47 expression contributes to pathogenesis by inhibiting phagocytosis of AML LSC. Consistent with this hypothesis, we found that increased CD47 expression predicted worse overall survival in three independent cohorts of adult AML patients. Furthermore, we predicted that disruption of the interaction of CD47 with SIRPα would result in phagocytosis and elimination of AML LSC. We found that blocking monoclonal antibodies directed against CD47 enabled phagocytosis of AML LSC—but not normal CD34+ human bone marrow progenitor cells—by human macrophages in vitro. Additionally, coating of human AML LSC with anti-CD47 monoclonal antibodies inhibited their engraftment in vivo in a xenotransplantation assay. Finally, analogous to a clinical therapy, treatment of human AML-engrafted mice with anti-CD47 antibody eliminated AML cells in the peripheral blood and bone marrow. In summary, increased CD47 expression is an independent poor prognostic factor that can be targeted on human AML stem cells with monoclonal antibodies capable of stimulating phagocytosis and elimination of LSC. Targeting CD47 with blocking monoclonal antibodies to induce phagocytosis is a novel mechanism for antibody cancer therapy. While anti-CD47 antibodies can be effective monotherapy for human AML, such antibodies may be equally, if not more, effective as part of a combination strategy. The combination of an anti-CD47 antibody, able to block a strong inhibitory signal for phagocytosis, with a second antibody able to bind an LSC-specific molecule and engage Fc receptors on phagocytes, thereby delivering a strong positive signal for phagocytosis, may result in a synergistic stimulus for phagocytosis and specific elimination of AML LSC.

18 Translational Biology of Cancer Stem Cells in Multiple Myeloma

William Matsui, Carol Ann Huff, Qiuju Wang, Ivan Borello, Richard J. Jones

Sidney Kimmel Comprehensive Cancer Center at The Johns Hopkins University, Baltimore, MD

Cancer stem cells (CSC) have been hypothesized to play a key role in disease initiation, maintenance, and relapse, but little data actually exist that CSC are clinically relevant, and few attempts have been made to target these cells in the clinic. We have studied multiple myeloma (MM) and found that the mature plasma cells that form the bulk of the tumor are incapable of clonogenic growth either in vitro or in vivo. In contrast, phenotypic memory B cells clonally related to the plasma cells can produce tumor colonies in vitro or disease in NOD/SCID mice. Moreover, MM CSC express mechanisms that protect normal stem cells from toxic injury and are resistant to agents currently used to treat MM patients. In contrast, the anti-B cell antibody rituximab has little activity against plasma cells but inhibits MM CSC in vitro. Based on these data, we initiated a Phase II clinical trial targeting MM CSC. Since mature plasma cells lack CD20, we combined agents that target plasma cells (cyclophosphamide [Cy]) and MM CSC (rituximab). We treated 21 patients and observed a median time to progression of 193 days. The median overall survival has not been reached with a median followup of 883 days, and two patients remain progression-free (654–1033 days). We serially quantified in vitro clonogenic MM growth prior to treatment, following count recovery and then at 2, 3, 6, 9, and 12 months. In all patients, MM colonies initially decreased. However, in 12 of 16 patients, MM colonies returned to baseline levels during the 12 months post-Cy that we carried out these correlative studies; all of these patients subsequently progressed. Interestingly, the return of myeloma CSC levels to baseline levels always preceded clinical progression by an interval of 30–120 days. Furthermore, the progression-free survival of these patients was significantly shorter than the patients whose CSC levels did not return to baseline during the year post-Cy in which these correlative studies were carried out. Since the clinical efficacy observed was modest, we also studied circulating myeloma stem cells from trial patients and found that they bound rituximab but were not cleared by the antibody. Thus, CD20 is a potential MM CSC target. We are currently planning a second-generation clinical trial utilizing radiolabeled anti-CD20 monoclonal antibodies. Moreover, clonogenic MM growth may serve as a novel biomarker that provides evidence for the clinical relevance of CSC and allows serial quantification of CSC during novel targeting trials.

19 New Epigenetic Drugs and Drug Combinations for Cancer Treatment

William G. Nelson, Zachery Reichert, Traci Speed, Andre Bergman, Xiaohui Lin, Srinivasan Yegnasubramanian
Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD

Epigenetic gene silencing ubiquitously accompanies the development of prostate cancer and other human cancers; reactivation of silenced genes has emerged as a rational treatment strategy. We have discovered several small molecule inhibitors of the 5-meCpG-binding domain proteins (MBDs) responsible for assembling transcriptional repression complexes at methylated CpG sequences near somatically silenced genes. The inhibitors, which appear to prevent the binding of MBDs to 5-meCpG-containing DNA, can synergize with nucleoside analog DNA methyltransferase (DNMT) inhibitors to reactivate certain silenced genes. As a group, the growing family of epigenetic drugs, including small molecule inhibitors of DNMTs, histone deacetylases (HDACs), and MBDs, triggers the reactivation of approximately 400 genes or more in cancer cells and changes the chromatin structure of many other genes in such a way as to facilitate expression in response to signaling and/or stress pathways. Data collected thus far have revealed that the new phenotypes induced in prostate cancer cells by epigenetic drugs expose unforeseen vulnerabilities to drugs targeting the products of reactivated genes. For this reason, the efficacy of epigenetic drugs in cancer may not be limited to reactivation of silenced tumor suppressor genes; rather, epigenetic drugs may also augment the activity of selected targeted drugs for cancer treatment. To explore the generality of this approach, we have attempted to use a combination of drug screening and epigenetic profiling approaches to discern the most promising epigenetic drug/targeted drug combinations, along with molecular biomarkers of treatment response, for clinical development for prostate cancer. Similar approaches can be considered for other human cancers.

20 Regulation of Gene Expression by Sphingosine in a Mouse Cell Model for Progressive Ovarian Cancer

Eva M. Schmelz, Amy L. Creekmore, and Paul C. Roberts
Virginia Polytechnic Institute and State University, Blacksburg, VA

Ovarian cancer is a disease of older women, often detected only as advanced disease. Despite tremendous efforts to prevent or cure ovarian cancer, its incidence and high mortality have remained largely unchanged. There are no proven methods of early prevention, and chemotherapeutic agents often are highly cytotoxic and cause severe side effects. We have generated new mouse ovarian surface epithelial (MOSE) cell lines that undergo sponaneous transformation and go through distinct stages that mimic early stages of ovarian cancer and that will subsequently progress to intermediate and, finally, late stages with a highly aggressive, malignant phenotype both in vitro and in vivo in immunocompetent mice. Using a total mouse genome cDNA (Affimetrix) array approach, we have now a list of genes aberrantly expressed during MOSE progression as either an early or late event. These genes were translated into functional groups and ranked for their importance for cancer progression using gene ontology tools; significantly overrepresented genes were found in cytoskeletal organization / regulation and metabolism. By comparison with 5'azaDC-treated cells, we have identified a list of genes that are epigenetically silenced during progression and, as such, are potential targets of dietary chemopreventive / chemotherapeutic compounds. We are interested in the effects of non-toxic concentrations of sphingosine, a bioactive sphingolipid metabolite that is generated not only in cells as a lipid second messenger, but also in the intestinal tract after hydrolysis of dietary complex sphingolipids; the regulation of aberrantly expressed genes; and the reversal or prevention of epigenetic silencing. We are currently confirming the expression levels of a list of genes that are significantly re-expressed by both sphingosine and 5'azaDC treatment and plan to use these genes for the investigation of the underlying mechanisms. Our long-term goal is to understand the specific regulation of events in transformed cells by non-toxic doses of sphingolipids and apply this knowledge to cancer prevention and treatment with orally administered complex sphingolipids.

21 Translating Developmental Regulators for Targeted Therapy in Pancreatic Cancer

Nelson S. Yee

Holden Comprehensive Cancer Center, Carver College of Medicine, University of Iowa

Histone deacetylases (HDACs) and RNA polymerase III (Polr3) are implicated in the control of cellular proliferation that is critical for normal development and neoplastic transformation. We present evidence that combination of an HDAC inhibitor (trichostatin A) and a small molecule inhibitor of Polr3 synergistically prohibited expansion of exocrine pancreas in zebrafish larvae. Combination of the clinical HDAC inhibitor (suberoylanilide hydroxamic acid, SAHA) with the Polr3 inhibitor produced supra-additive suppression of proliferation and induction of apoptosis in human pancreatic adenocarcinoma cells. The enhanced cytotoxicity is partially attributed to the SAHA-stimulated expression of tRNAs being reversed by the Polr3 inhibitor. These findings demonstrate early translation of developmental studies in zebrafish to human cancer and suggest combined targeting of developmental regulators as a potential approach to improve treatment response and overcome therapeutic resistance in pancreatic cancer. We will test the in vivo anti-tumor and toxic effects of the small molecules combination in animals, and we plan for future clinical trials in patients with pancreatic adenocarcinoma to determine the efficacy and safety of the combination of SAHA and the Polr3 inhibitor.

22 Discovery of Novel Molecular Profile of Response to Cetuximab Using a Phosphoproteomic Approach

Aarif Ahsan[1], Xiaoping Ao[1], David M. Lubman[2], Theodore S. Lawrence[1], **Mukesh K. Nyati**[1]

[1]Department of Radiation Oncology, [2]Department of Surgery, University of Michigan, Ann Arbor

With recent discoveries into the genetics of cancer, many new targets for cancer treatment have been identified. One of these targets is the EGFR, which is amplified or overexpressed in various types of cancers, including those of head and neck origin. Along with genetic discoveries, several new agents have been developed in an effort to target these molecules with the hope of achieving cure without the toxicity typically associated with traditional chemotherapeutic agents. One such agent, cetuximab, has been proven to be effective in local and metastatic head and neck cancers in combination with cisplatin or radiation. Although these results are encouraging, still only one out of four patients treated with cetuximab in combination with radiation or cisplatin benefits from this novel and very expensive treatment. We feel it is of great importance to differentiate between patients who are likely to respond to cetuximab and those who are not. EGFR immunostaining in pre-treatment patient specimens and analysis of EGFR gene copy number have failed to predict response to EGFR inhibition therapy. We hypothesize that through phosphoproteome analysis we can identify alterations in novel signal transduction molecule(s) induced by cetuximab treatment that will predict subsequent response to cetuximab. To this end, we assessed phosphoproteomic changes upon cetuximab treatment in UMSCC-1 (cetuximab-responsive) and UMSCC-74 (cetuximab-non-responsive) cell lines. We found pharmacodynamic changes in over 12 novel proteins upon cetuximab treatment in UMSCC-1 cell line. We have initially investigated three novel proteins (NCoR1, MeCP2, and MBD2), as they are part of the large family of methyl-CpG binding proteins and cause transcriptional repression (NCoR1 and MeCP2) or activation (MBD2) by immunoprecipitating total proteins followed by immunoblotting with phospho-specific antibodies. Using this strategy, we have confirmed (a) that these three proteins are indeed phospho proteins, (b) in UMSCC-1 cells phosphorylation of both NCoR1 and MeCP2 is induced and phosphorylation of MBD2 is decreased, confirming phosphoproteomic data. Importantly, in UMSCC-74B we found that cetuximab caused either no effect or an opposite effect to that seen in the sensitive cells. These data suggest that we have a powerful strategy for finding novel biomarkers of response to cetuximab. These biomarkers and additional biomarkers that we hope to discover will undergo rigorous in vitro and in vivo testing before we employ them on TMA that we are currently generating from a xenograft study.

23 Hedgehog-GLI and EGF Pathway Interaction in Pancreatic Cancer

Luciana L. Almada, Sherine Elsawa, Jing-Jing Han, George Lund, Julieta Peña-Sanchez, Charles Erlichman, Martin E. **Fernandez-Zapico**

Schulze Center for Novel Therapeutics, Division of Oncology Research, Mayo Clinic, Rochester, MN

Interactions between oncogenic pathways are key molecular events in the initiation, progression, and maintenance of the transformed phenotype. Several studies suggest that the ability of the EGF pathway, an oncogenic cascade that is activated in more than 90% of pancreatic tumors, to transform cells depends on cooperation with other oncogenic pathways. However, the potential EGF-interacting oncogenic pathways, as well as the molecular mechanisms underlying this phenomenon in pancreatic cancer cells, remain elusive. Therefore, further characterization of EGF signaling interactions will be crucial for the understanding of the complex and varied networks of signaling cascades that leads to pancreatic neoplastic transformation. Here, we show evidence of a molecular interaction between EGF and Hedgehog (HH)-GLI pathways, a novel pancreatic carcinogenic cascade. Specifically, we demonstrate the ability to EGF to activate GLI transcription factors, essential effectors of the HH oncogenic function in pancreatic cancer cells. Expression and luciferase reporter assay in combination with genetic and pharmacological manipulations of the EGF pathway show that this cascade is able to activate GLI transcriptional activity, even in the absence of an active HH signaling. Further analysis of this molecular interaction suggests that EGF-mediated activation of GLI transcription factors requires an intact PI3K pathway. Finally, sequence analysis identifies candidate EGF-dependent phosphorylation sites within GLI domains that are essential for their transcriptional activity, thus, suggesting potential molecular mechanisms underlying the modulation of GLI function by EGF in pancreatic cancer cells. Taken together, these results provide a novel insight into the complex network involved in pancreatic carcinogenesis and present new avenues for the development of therapeutics regimens for this dismal disease.

24 Targeting HER3 in HER2-Positive Breast Cancers

Rebecca S. Cook[1,2], Joan Garrett[2,3], Cammie Rinehart[3], Carlos L. Arteaga[1,2,3]

[1]Department of Cancer Biology; [2]Vanderbilt-Ingram Cancer Center; [3]Department of Medicine, Vanderbilt University, Nashville, TN

The ErbB family of receptor tyrosine kinases (RTKs) includes EGFR/ErbB1, HER2/ErbB2, kinase-inactive HER3/ErbB3, and HER3/ErbB4. Expression and activation of EGFR and HER2 in the mammary epithelium contributes to cellular proliferation, and their overexpression is causally associated with breast cancer formation and increased malignancy. Increasing evidence suggests that heterodimerization of HER3/ErbB3 with HER2 increases HER2-induced proliferation and transformation through increased phosphotidyl inositol 3-kinase (PI3K) signaling to Akt. We have developed a mouse model in which ErbB3 gene expression in inhibited specifically in the mammary epithelium using Cre-mediated genetic recombination. Delayed lengthening of the ductal epithelium due to increased cell death and decreased cellular proliferation was observed in response to loss of ErbB3 and correlated with decreased Akt phosphorylation in mammary epithelial cells. Loss of ErbB3 resulted in an accumulation of stem cells and basal cells at the expense of mature luminal epithelial cells, accompanied by increased stromal cellularity and collagen deposition surrounding TEBs. Taken together, these results suggest that ErbB3 is required to sustain luminal epithelial cells. Using the MMTV-PyVmT transgenic mouse model of breast cancer, a model that harbors HER2+ tumors, we found that loss of ErbB3 impairs tumor formation and metastasis to lungs. Tumor cell proliferation was decreased due to loss of ErbB3, and a vast increase in the level of cell death was seen in ErbB3-deficient tumors. Activation of PI3K, as measured by phosphorylation of its downstream target, Akt, was decreased in PyVmT-induced tumors lacking ErbB3, similar to what was seen in tumors from mice treated with the HER2 tyrosine kinase inhibitor, lapatinib. These data suggest that HER3/ErbB3 is a critical mediator of HER2-directed growth, survival, and transformation of the breast epithelium.

25 Reverse-Phase Protein Array (RPPA) Identification of Epithelial Markers Associated With Response to Dasatinib (D) and/or Erlotinib (E) Treatment in Head and Neck Squamous Cell Carcinoma (HNSCC)

Ann Marie Egloff[1], Priya Koppikar[2], Dan Shiwarski[1], Brian Trevelline[1], Doris Siwak[2], Gordon Mills[2], Jennifer R. Grandis[1]

[1]Department of Otolaryngology, University of Pittsburgh, Pittsburgh, PA; [2]Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York; [3]Department of Systems Biology, The University of Texas M. D. Anderson Cancer Center, Houston

Purpose: To identify proteins/phosphoproteins in head and neck sqamous cell carcinoma (HNSCC) cell lines whose levels correlate with response to Src-inhibition and/or combined Src-EGFR- inhibition by RPPA and to interrogate HNSCC tumor RPPA data for possible mechanisms of response/resistance. Background: EGFR-targeting tyrosine kinase inhibitors (TKIs) are in clinical development for the treatment of HNSCC with modest results. Combining Src-targeting TKIs with EGFR-inhibitors is hypothesized to improve clinical response. Identifying candidate proteins/phosphoproteins whose levels in HNSCC cell lines differentiate those that are responsive versus resistant to combined EGFR- and Src-inhibition is a first step towards defining HNSCC tumors that are more likely to respond to this combination treatment. Methods: HNSCC cell viability was assessed by MTS assay following 72 hour treatment with varying concentrations of E and/or D TKI, targeting EGFR and Src kinases, respectively; IC-50 values were calculated using PRISM software. Relative protein and phosphoprotein levels in 4 HNSCC cell lines and 69 HNSCC tumors were determined by nonparametric algorithm following RPPA with computerized optical density detection quantification and validated by immunoblot in a panel of nine HNSCC cell lines. Results: E-cadherin levels were significantly reduced in HNSCC cells resistant to D ($p=0.016$), defined as having an IC-50 value greater than 200nM. Vimentin levels were higher in D-resistant HNSCC cell lines ($p=0.016$). IC-50 values for D and E were highly correlated (Spearman's rho=0.904, $P=0.002$). Combined treatment with 100nM D and 2uM E for 6 days resulted in significant ($P<0.05$) though modest enhanced cell death compared to treatment with either agent alone in two vimentin-expressing HNSCC cell lines. In HNSCC tumors, E-cadherin levels were positively correlated with EGFR P-Y992 and HER2 P-Y1248 levels ($P=0.008$ and <0.001, respectively), while c-Met levels tended to be inversely correlated ($P=0.036$). Conclusions: Our data indicate that HNSCC cell lines exhibiting epithelial characteristics are more sensitive to D or E in vitro. Epithelial-mesenchymal transition (EMT) has been reported to be associated with resistance to EGFR TKIs. Combining D with E enhanced antitumor effects in cell lines exhibiting EMT ($P<0.05$), suggesting that HNSCC with EMT molecular characteristics may be responsive to combined Src- and EGFR-targeted treatments. HNSCC tumor data suggest that EGFR/HER2 activation may be associated with response, while upregulation of c-MET may be associated with resistance to D and/or E treatment.

26 Phase I Trial of GDC0449 and Erlotinib

Charles Erlichman, Martin Fernandez-Zapico, Wilma Lingle, George Kim, Donald Northfelt, Thomas Smyrk, Val Lowe
Mayo Clinic, Rochester, MN

We have demonstrated that crosstalk between the Hedgehog-GLI and EGFR signaling pathway exists and contributes to pancreatic tumorigenesis. This interaction can be abrogated by targeting both pathways simultaneously and results in a synergistic antitumor effect in vitro and in vivo in pancreatic tumor models. Based on these results we have undertaken a phase I trial of GDC0449 (an orally administered Hedgehog inhibitor) and Erlotinib (EGFR tyrosine kinase inhibitor to: (1) determine the maximally tolerated dose (MTD) of GDC-0449 combined with Erlotinib in unresectable solid tumors; (2) describe the adverse events associated with this treatment combination; (3) describe the responses of this treatment combination; and (4) assess the effect of the Erlotinib and GDC-0449 combination on selected biomarkers in circulating tumor cells (CTCs), tumor biopsies, and FDG-PET in a cohort of patients with metastatic pancreatic cancer treated at the MTD. During the dose escalation portion of the trial, eligible patients receive GDC0449 150 mg p.o. daily and Erlotinib 50, 75, 100, or 150 mg daily p.o. at dose levels 1, 2, 3, and 4 respectively. Toxicity assessment is performed at each treatment cycle and response assessment every second treatment cycle, which is defined as 28 days. At the MTD, up to 20 patients with previously untreated metastatic pancreatic cancer will be entered to assess the biologic effect of GDC0449. Patients will be treated with GDC0499 alone for one cycle. Pre- and post-treatment biopsies of metastases and FDG-PET will be performed. Weekly collection of CTCs will be undertaken, and immunofluorescence microscopy of total and phosphoMAPK, EGFR, AKT, PATCHED1, GLI1, BCL-2, and quantitative PCR (Q-PCR) of PATCHED1, GLI1, BCL-2, BFL-1/A1, 4-1BB will be performed. Total and phosphoMAPK, EGFR, AKT, PATCHED1, GLI1, BCL-2 by immunohistochemistry and Q-PCR of PATCHED1, GLI1, BCL-2, BFL-1/A1, 4-1BB will be analyzed in tumor biopsies. Currently we are accruing at dose level 2. No significant toxicities have been observed. Supported by CA69912 and CA102701.

27 Vandetanib Induced Reduction in Phosphorylation of AKT Is a Biomarker of Drug Sensitivity in HNSCC Cell Lines

Mitchell J. Frederick, Genevieve A. Andrews, Mei Zhao, David R. Fooshee, Zvonimir L. Milas, Maria Gule, Chad E. Galer, Lauren A. Byers, John V. Heymach, Scott M. Lippman, **Jeffrey N. Myers**
The University of Texas M. D. Anderson Cancer Center

Overexpression of the epidermal growth factor receptor (EGFR) is commonly observed in primary squamous cell carcinomas of the head and neck, as well as lung. Aberrant signaling through the EGFR is thought to activate proliferation and survival in these cancers, yet paradoxically drugs inhibiting the EGFR (EGFRi) have had limited clinical success as single agents in head and neck squamous cell carcinomas (HNSCC), compared to lung cancers. Therefore, drugs that target angiogenesis in addition to EGFR signaling, such as Vandetanib, may be more useful for treating HNSCC patients. Given the low rates of clinical response to EGFRi alone, however, it remains unclear whether these dual kinase inhibitors would be more advantageous than drugs exclusively targeting angiogenesis receptors. To clarify the role of EGFR signaling, as well as examine mechanisms and biomarkers defining EGFRi sensitivity, we investigated the ability of Vandetanib to inhibit growth in a panel of 47 different HNSCC lines in vitro where the principal drug effects are presumably through the EGFR. The GI50 values for cell lines ranged from 0.4 μM to 14 μM, representing at least a log-fold concentration difference between the most sensitive and resistant lines. When sensitive HNSCC lines FADU and UMSCC10B were treated for 30h with 4μM Vandetanib in the presence of serum, there was a highly significant reduction in phosphorylation of AKT on Ser427. In contrast, no such reduction in pAKT Ser427 was observed for two resistant cell lines examined, SCC61 and JHU 028. Interestingly, there was also a parallel reduction in constitutive pERK in the two sensitive cell lines following 30h of Vandetanib drug treatment, which only occurred in one of the resistant lines, SCC61. EGF-stimulated phosphorylation of the EGFR was equally inhibited in both resistant and sensitive cells by 4μM Vandetanib, indicating that resistance was not due to a difference in drug target sensitivity. Collectively, our preliminary data suggests a model whereby sensitive cells rely heavily upon the EGFR pathway to activate and maintain survival mechanisms such as phosphorylation of AKT, whereas resistant cells probably use other receptor or cell signaling pathways to achieve the same end.

28 Tamoxifen, HER2, and Endoxifen: The Role of CYP2D6 as a Predictor of Tamoxifen Resistance in ER+/HER2+ Breast Cancer

Matthew P. Goetz[1,2], Kathryn E. Reinicke[2], Joel Reid[1,2], Vera J. Suman[3], Mary Kuffel[2], Stephanie Safgren[2], Sarah Buhrow[2], Carol A. Reynolds[4], Robert Jenkins[4], John Hawse[5], Edith A. Perez[6], James N. Ingle[1], Matthew M. Ames[1,2]

Departments of [1]Oncology, [2]Molecular Pharmacology and Experimental Therapeutics, [3]Biostatistics, [4]Pathology, [5]Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN; [6]Division of Hematology/Oncology, Mayo Clinic, Jacksonville, FL

Background: Endoxifen is undergoing development for estrogen receptor (ER) + breast cancer (BC), including National Cancer Institute production of clinical grade drug, preclinical toxicology/pharmacology, and materials for IND submission. HER2 expression in ER+ BC is associated with tamoxifen resistance, and tamoxifen administration to mice bearing ER+/HER2+ xenografts stimulates BC growth (Shou, JNCI 2004). Endoxifen degrades ER alpha and is the most important tamoxifen metabolite responsible for inhibiting BC growth (Wu, Cancer Research 2009). CYP2D6 metabolism affects endoxifen concentrations (Stearns, JNCI, 2003) and is associated with recurrence in tamoxifen-treated BC (Goetz, JCO, 2005). We sought to determine the activity of tamoxifen and metabolites in ER+/HER2+ BC cell lines and to evaluate the role of CYP2D6 metabolism in tamoxifen-treated patients (pts) with ER+/HER2+ BC. Additionally, we determined endoxifen concentrations in mice administered oral tamoxifen. Methods: MCF7 (parental and HER2-expressing) and BT474 (ER+/HER2+) cells were used to compare the activity of tamoxifen, 4HT, and endoxifen on estrogen-stimulated growth. Oral tamoxifen PK were characterized in mice treated with standard dose tamoxifen (4 mg/kg). Clinical data were obtained via a retrospective analysis of tamoxifen-treated patients with ER+/ HER2+ BC randomized to 5 years of tamoxifen (NCCTG 89-30-52). CYP2D6 metabolism (extensive or decreased) was based on CYP2D6 genotype (*3, 4, 6, 10, 17, 41) and co-administration of a CYP2D6 inhibitor (yes/no). HER2 was determined by IHC or FISH. The association between CYP2D6 and DFS was assessed using the log-rank test and proportional hazards modeling. Results: Compared to tamoxifen, endoxifen potently inhibited the growth of BT474 cells. In MCF7 cells, expression of HER2 shifted the endoxifen IC50 from 54 nM (parental) to 131 nM (HER2-expressing). Using concentrations of tamoxifen and metabolites observed in humans, only endoxifen potently inhibited the growth of MCF7HER2—but only at concentrations achievable in CYP2D6-extensive metabolizers (>50nM). In mice, 4HT and endoxifen concentrations were below 15 nM following oral tamoxifen. In NCCTG 89-30-52, HER2 was expressed in 23/215 (11 percent) but not associated with DFS (p=0.62). In HER2+ subset, patients with decreased CYP2D6 metabolism (n=10) had significantly shorter DFS compared to extensive metabolizers (n=9) (HR 9.5, p=0.03). Conclusions: Our data provide a simple pharmacological model for understanding HER2 resistance in tamoxifen-treated BC. Mice bearing BC xenografts should not be used to model tamoxifen resistance given that they lack CYP2D6. Our data support the ongoing development of endoxifen and suggest that endoxifen may overcome de novo tamoxifen resistance in ER+/HER2+ BC.

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29 Novel Therapeutic Targets in TCGA Cancer Sequencing Data

Heidi Greulich, Milan Chheda, Tzu-Hsiu Chen, Kumiko Tanaka, Amit Dutt, Kwok-Kin Wong, William Hahn, Matthew Meyerson

Dana-Farber Cancer Institute and Broad Institute of the Massachusetts Institute of Technology and Harvard University

The Cancer Genome Atlas (TCGA) Consortium recently completed a multidimensional analysis of glioblastoma, characterizing somatic alterations across a large sample set and identifying genetic lesions that likely contribute to tumor fitness (TCGA Network, Nature, 2008). Such “driver” genes may also represent therapeutic opportunities if tumor cells are dependent on the aberrant gene function for survival, a principle known as “oncogene addiction”. We have begun target validation on selected receptor tyrosine kinase mutations reported in the TCGA glioblastoma publication. We have found that a subset of reported mutations in the receptor tyrosine kinases ERBB2 and FGFR3 are gain-of-function, oncogenic, and sensitive to specific small molecule kinase inhibitors. In the case of ERBB2, several of these inhibitors are in Phase II clinical trials or beyond for other cancers and may be redeployable for glioblastoma patients who harbor ERBB2 mutations.

30 Efficacy of Combined Vandetanib, Docetaxel, and Radiation Therapy in Human HNSCC Xenografts: Effect of EGFR Status and Utilization of Pharmacokinetic-Directed Dosing

Daniel L. Gustafson[1,2], Erica L. Bradshaw-Pierce[2]

[1]Animal Cancer Center, Colorado State University, Fort Collins, CO; [2]University of Colorado Comprehensive Cancer Center, University of Colorado, Denver, Aurora, CO

Vandetanib (ZD6474; Zactima®) is a multi-target tyrosine kinase inhibitor (TKI) with activity against VEGFR2 and EGFR, thus exhibiting both antiangiogenic as well as direct antitumor effects. The pre-clinical development of TKIs often involves screening for activity in combination with cytotoxic therapies as well as the analysis of sensitive versus resistant cell lines to determine underlying molecular pathways indicative of response. The anti-EGFR activity of vandetanib dominates when human head and neck squamous cell carcinoma (HNSCC) cell lines are screened for anti-proliferative effects resulting in response similar to gefitinib. However, studies in xenograft models have shown that vandetanib can enhance anti-tumor response of radiation therapy (RT) even in HNSCC xenografts completely refractory to vandetanib therapy alone (UMSCC10), as well as in responsive HNSCC xenografts (UMSCC2). Since docetaxel (DTX) combined with RT is a common treatment for HNSCC, and the addition of EGFR inhibitors to HNSCC therapy has shown promise, we determined the effects of pharmacokinetic-directed, clinically equivalent dosing of vandetanib and DTX combinations with RT utilizing EGFR-positive (UMSCC2) and EGFR-null (UMSCC10) HNSCC xenografts. The purpose of these studies was to determine: (1) whether only EGFR-positive tumors benefit from vandetanib addition to treatment combinations; (2) the combined effects of therapies at putative clinical doses for vandetanib and DTX; and (3) whether vandetanib/RT combinations were sufficiently equivalent to DTX/RT combinations in these xenograft models to support testing of this potentially less toxic therapeutic combination. The results from these studies show that vandetanib can enhance the effects of RT and RT/DTX in both UMSCC2 and UMSCC10 tumors and that robust anti-tumor response can be observed utilizing drug doses that mimic human exposures at weekly 30 mg/m2 for DTX and 100–300 mg daily doses of vandetanib. Vandetanib/RT versus DTX/RT treatment combinations showed equivalent effects in the UMSCC2 and UMSCC10 tumors. The results from these studies show that both EGFR-positive and EGFR-null tumors show enhanced response when vandetanib is combined with DTX and RT at clinically relevant drug doses. Further, the results from these studies suggest that vandetanib/RT is equally efficacious as DTX/RT and vandetanib/DTX/RT in EGFR-positive and EGFR-null tumors and that potential decreased toxicity of RT combinations lacking a taxane component may warrant further study.

31 Design and Development of Inhibitors of the Ubiquitin E3 Ligase BCA2 for the Treatment of Breast Cancer

Fathima R. Kona[1], Daniela Buac[1], Andrew Westwell[2], Arun K. Seth[3], **Angelika M. Burger**[1]

[1]Barbara Ann Karmanos Cancer Institute, Wayne State University, Detroit, MI; [2]Cardiff University, Welsh School of Pharmacy, Cardiff, United Kingdom; [3] Sunnybrook Research Institute, Toronto, ON, Canada

Ubiquitin E3 ligases have been implicated in the pathogenesis of breast cancer. They control critical signaling pathways. RING-type E3 ligases regulate the stability of tumor suppressor proteins and oncogenic receptor tyrosine kinases, including HER2 and EGFR. We have cloned a RING-finger ubiquitin E3 ligase breast cancer associated gene 2 (BCA2) from invasive breast cancer cells and found it overexpressed in 56% of invasive breast cancers. A binding partner of BCA2 is Rab7, a small GTPase that is involved in receptor endocytosis and recycling. Rab7 regulates endocytic trafficking of the EGF/EGFR complex. Targeting BCA2 with small molecule inhibitors could be a novel approach to anti-EGFR therapy. We reasoned that compounds that can modify the catalytic activity of BCA2 would be good candidate inhibitors. The RING-finger complexes two Zn2+ ions that are pivotal for BCA2 function. Thus, we tested a series of 10 zinc-ejecting compounds from the National Cancer Institute including NSC25953 (disulfiram, DSF). DSF inhibited BCA2 ligase activity with a potency similar to BCA2 siRNA. This translated into in vitro and in vivo growth inhibition of breast cancer cells expressing BCA2 such as T47D, MCF-7, and MCF-7 clones resistant to tamoxifen or trastuzumab but not those cells lacking BCA2 (MDA-MB-231). DSF is an approved drug for the treatment of alcoholism. It blocks aldehyde dehydrogenase (ALDH) and generates toxic metabolites upon alcohol ingestion. Because of the latter, we designed three novel series of disulfide compounds with the intent to find more potent analogs lacking ALDH inhibition. Dithiocarbamates (34), carbamo(dithioperoxo)thioates/DPTs (10), and disulfides (4) were synthesized and tested for cytotoxicity in MCF-7 and MDA-MB-231 breast cancer cell lines; ALDH inhibition was also assessed. While dithiocarbamates had no antiproliferative activity, 9/10 DPTs were cytotoxic to BCA2+ and BCA2- cells, and DPT003 was active in MCF-7 cells only (IC50=900nM). The disulfides (D24, D12, and D17) showed in vitro antiproliferative activity in MCF-7 cells (D24 IC50=30nM), but none (D24) or less (D12<D17) in MDA-MD-231 cells. All compounds with activity in MCF-7 cells were able to inhibit the ligase activity of recombinant BCA2. However, only D12 and DPT003 were devoid of anti-ALDH activity. Our data suggest that the disulfanyl methanethione group is important for E3 ligase inhibition; DPT003 and D12 might be specific inhibitors of BCA2 and should undergo further preclinical testing.

32 Mechanism of Radiosensitization by Molecularly Targeting Growth Factor Receptors

Raymond E. Meyn[1], Anapama Munshi[1], John V. Haymach[2], Luka Milas[1], K. Kian Ang[3]

Departments of [1]Experimental Radiation Oncology, [2]Thoracic/Head and Neck Medical Oncology, and [3]Radiation Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX

Cancers of the upper aerodigestive tract (UADT) are especially problematic in human health. Lung cancer is the leading cause of deaths due to cancer in the United States, and head and neck squamous cell carcinoma (HNSCC), another UADT tumor, is the fifth most common cancer in the United States. Despite improvements in treatment strategies, local/regional control remains a problem, indicating that further advances in treatment are urgently needed. This situation has prompted investigations into the reasons that would explain resistance to intensive combined-modality therapies. One of the important outcomes of this research has been the recognition that the majority of lung tumors, especially non-small cell lung cancer (NSCLC), and HNSCC tumors abnormally express the epidermal growth factor receptor (EGFR). EGFR is a member of a family of growth factor receptors collectively referred to as receptor tyrosine kinases (RTKs). Based on this appreciation of EGFR's role in these cancers, several molecularly targeted agents have been developed to inhibit the activity of this growth factor receptor, including gefitinib, erlotinib, and cetuximab. In addition to their well-established clinical activities, these agents are all, at least in preclinical models, radiosensitizers for a variety of tumor types including NSCLC and HNSCC. The radiosensitizing effects of these EGFR antagonists correlates with a suppression of the ability of the cells to repair radiation-induced DNA double strand breaks (DSBs). However, the molecular mechanisms by which EGFR govern DNA repair capacity is multifactorial and appears to be mediated by one or more signaling pathways downstream of such receptors. Investigations to date have focused on two pathways in particular (i.e., the PI3K/AKT and the Ras/Raf/MEK/ERK pathways). However, we have recently discovered that the epithelial-to-mesenchymal transition (EMT) status of NSCLC and HNSCC cell lines also dictates their inherent radiosensitivity and ability to be radiosensitized by EGFR antagonists. This revelation suggests that biomarkers for EMT status could be used to identify patients that would benefit from combinations of EGFR antagonists and radiation and points to new pathways that could be exploited for enhancing patient response to radiotherapy. Supported by PO1 CA06294 from the National Cancer Institute.

33 Mechanisms of Sensitivity and Resistance to EGFR Tyrosine Kinase Inhibitors in Lung Cancer

William Pao

Vanderbilt-Ingram Cancer Center, Department of Medicine, Vanderbilt University, Nashville, TN

The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) gefitinib (Iressa) and erlotinib (Tarceva) induce dramatic responses in certain patients with non-small cell lung cancer (NSCLC). During the past few years, we have defined biomarkers that predict primary sensitivity, primary resistance, and secondary (or acquired) resistance to these drugs. A major predictor of primary sensitivity is EGFR kinase domain mutations, which are composed predominantly of recurrent deletions in exon 19 and a recurrent point mutation in exon 21 (L858R). Markers of primary resistance include mutations in genes that encode signaling proteins downstream of EGFR (i.e., KRAS and BRAF). Patients with acquired resistance to these drugs develop other types of genetic alterations. Tumors in about half of such patients harbor second-site EGFR kinase domain mutations. The most common (>90%) second-site mutation involves a point mutation in exon 20 (T790M). Rarer mutations include D761Y and T854A. Another 20% of patients harbor tumors with amplification of the gene encoding another tyrosine kinase, MET. MET amplification occurs with or without T790M mutations. Transgenic mice with inducible expression in type II pneumocytes of EGFRT790M alone or together with a drug-sensitive EGFRL858R mutation develop lung adenocarcinomas that require mutant EGFR for tumor maintenance but are resistant to erlotinib. We are now using these animal lung tumor models to identify potential therapeutic strategies to overcome EGFRT790M-mediated resistance. For example, we have found that the combination of a new EGFR TKI, BIBW-2992, in conjunction with cetuximab, an anti-EGFR antibody, is synergistic and extremely effective at inducing complete tumor responses in mice bearing EGFRT790M-driven lung tumors. In primary mouse lung tumors, xenografts, and fibroblast transfectants, only the combination of both agents together induced near complete depletion of both phosphorylated and total EGFR. Novel dual targeting with cetuximab and a "second-generation" EGFR inhibitor may be an effective strategy to overcome T790M-mediated resistance.

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34 Molecular Targeted Combinational Chemopreventive Drug Development: Promise and Progress for the Prevention and Treatment of Colorectal Cancer

Chinthalapally V. Rao[1], Ronald Lubet[2], Levy Kopelovich[2], Vernon E. Steele[2]

[1]Department of Medicine, Hematology-Oncology Section, University of Oklahoma Cancer Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK; [2]Chemopreventive Agent Development Research Group, Division of Cancer Prevention, National Cancer Institute

Clinical and preclinical studies suggest that molecular targeted small molecule drug development approaches are very promising at the preclinical and clinical levels for the prevention and treatment of several epithelial cancers. NSAIDs such as aspirin, sulindac, and celecoxib (a cyclooxygenase (COX)-2 inhibitor), DFMO (an ODC inhibitor), atorvastatin (a HMG-R inhibitor), Targretin (an RXR-modulator), and giftnib (an EGFR inhibitor) have been shown to reduce the risk of colon and other epithelial cancers. However, their prolonged administration at higher doses add significant efficacy but also cause for concern regarding unwanted toxicity. For example, celecoxib is highly effective in the prevention of colonic polyps, but its prolonged administration at higher doses increase cardiovascular risk, a major concern. Thus, developing different molecular targeted chemopreventive combinations may provide additive and synergistic efficacy at low doses, without any unwanted side effects. Using preclinical models of colon cancer, we have identified several promising combinational molecular targets and generated impressive data using both single and multiple agents. These targets include 5-Lipoxygenase (5-LOX)/COX-2; COX-2 and HMG-R; ODC and COX; p53 and COX-2; and RXR and ER-beta. Thus, development of agents with 5-LOX/COX inhibition or combinations of agents that represent different modes of action provide a practical approach for improving chemopreventive and therapeutic efficacy without unwanted side effects. For example, experiments were designed to test the chemopreventive/therapeutic efficacy of licofelone, a novel 5-LOX/COX-inhibitor. To test the efficacy of licofelone (50–300 ppm in diet) in the colon, we utilized an azoxymethane (AOM)-induced rat colon carcinogenesis and Apc-min intestinal tumor model and AOM-induced rat colon adenocarcinoma model. In the rat colon model, licofelone suppressed AOM-induced colonic aberrant crypt foci (ACF) in a dose-dependent manner (Total ACF, $p<0.0.05–0.0001$; multicrypt foci, $p<0.01–0.0001$); similarly, Apc-min intestinal tumor formation was also significantly suppressed ($p<0.01–0.0001$). We will discuss ongoing progress on the combinational targets, identification of select chemopreventive agents, and promise for the human clinical trials.

35 Clinical Features and Outcome of Patients With Non-Small Cell Lung Cancer Harboring ALK Translocations

Alice T. Shaw[1], **Daniel B. Costa**[2], Beow Y. Yeap[1], Mari Mino-Kenudson[1], Subba R. Digumarthy[1], Rebecca S. Heist[1], Benjamin Solomon[3], Hannah Stubbs[1], Sonal Admane[1], Ultan McDermott[1], Jeffrey Settleman[1], Susumu Kobayashi[2], Eugene J. Mark[1], Scott J. Rodig[4], Lucian R. Chirieac[4], Eunice L. Kwak[1], Thomas J. Lynch[1], A. John Iafrate[1]

[1]Massachusetts General Hospital; [2]Beth Israel Deaconess Medical Center, Harvard Medical School; [3]Department of Haematology and Medical Oncology, Peter MacCallum Cancer Centre, Melbourne, Australia; [4]Department of Pathology, Brigham and Women's Hospital

Purpose: EML4-ALK and other ALK fusion oncogenes represent a novel molecular target in a subset of non-small cell lung cancers (NSCLC). To aid in identifying and treating these patients, we examined tumor pathology, clinical characteristics, and treatment outcomes in advanced NSCLC patients with and without ALK translocations. **Patients and Methods:** Patients with NSCLC were selected for screening based on 2 or more of the following characteristics: female gender, Asian ethnicity, never/light smoking history, and adenocarcinoma histology. ALK translocations were identified using FISH (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe, Abbott Molecular) and confirmed by immunohistochemistry for ALK expression. EGFR and KRAS mutational statuses were determined by DNA sequencing. **Results:** Of 141 tumors screened, 19 (13%) were ALK-translocation-positive, 31 (22%) were EGFR-mutated, and 91 (65%) were wild-type (WT/WT) for both ALK and EGFR. None of the ALK- or EGFR-mutated patients had KRAS mutations. Compared to the EGFR-mutated and WT/WT cohorts, patients with ALK translocations were significantly younger (median age 66, 64, 52; P=0.005) and more likely to be male (26%, 32%, 58%; P=0.039). ALK-positive, like EGFR-mutated, NSCLC patients were also more likely to be never/light smokers compared with WT/WT patients (100% versus 43 percent; P<0.001). Some 18/19 ALK-translocation-positive tumors were adenocarcinomas, half with solid growth pattern and signet rings cells. Among stage IV patients, ALK translocation was associated with resistance to EGFR tyrosine kinase inhibitors (TKIs) with response rate (RR) of 0% (0/9) and median time to progression (TTP) of 5 months, while EGFR-mutated tumors were extremely sensitive with 70% at RR (16/23) and median TTP of 16 months (p=0.004). ALK-translocated, EGFR-mutated and WT/WT cases showed similar response rates to platinum-based chemotherapy (RR 25%, 50%, 35%; p=0.356) and TTP (approximately 8–10 months). **Conclusion:** ALK translocations (in specific EML4-ALK) define a molecular subset of NSCLC with distinct clinical and pathologic characteristics. Patients harboring ALK fusions do not benefit significantly from EGFR TKIs and should be treated with other standard agents or ALK targeted therapies. Initial impressive RRs exceeding 50% have been seen with a novel ALK/MET TKI PF-02341066 in ALK-translocated NSCLC.

36 Preclinical Assessment of Dual ErbB1/ErbB2 Targeting in Intrahepatic Cholangiocarcinoma

Alphonse E. Sirica, Zichen Zhang, Deanna J.W. Campbell

Division of Cellular and Molecular Pathogenesis, Department of Pathology, Virginia Commonwealth University School of Medicine, Medical College of Virginia Campus, Richmond, VA

Aberrant ErbB signaling has been implicated in the pathogenesis of intrahepatic cholangiocarcinoma, suggesting that targeting strategies utilizing specific ErbB receptor tyrosine kinase inhibitors might prove to be an effective neo-adjuvant therapy for this lethal cancer. To test this strategy, we investigated targeting with the ErbB1 inhibitor tryphostin AG1517 and the ErbB2 inhibitor tryphostin AG879, in combination and alone, as well as with the dual ErbB1/ErbB2 inhibitor lapatinib (GlaxoSmithKline), to assess the effectiveness of simultaneous targeting of ErbB1 and ErbB2 signaling over single inhibitor treatments in suppressing cholangiocarcinoma cell growth in vitro and the therapeutic efficacy of lapatinib in vivo. Our in vitro studies were carried out using two rat (BDEneu and C611B) and three human (HuCCT1, EGl1, and TFK1) cholangiocarcinoma cell lines. The efficacy of lapatinib to significantly suppress liver tumor growth was tested in an orthotopic, syngeneic rat model of intrahepatic cholangiocarcinoma progression. Our results demonstrated that simultaneous targeting of ErbB1 and ErbB2 was significantly more effective in suppressing the in vitro growth of both rat and human cholangiocarcinoma cells than individual receptor targeting. Mechanistically, tryphostin AG1517 combined synergistically with tryphostin AG879 to significantly inhibit rat BDEneu and C611B cell growth in culture via enhanced inhibition of ErbB signaling. Dual targeting with AG1517 and AG879 also markedly suppressed cyclooxygenase-2 expression in cultured cholangiocarcinoma cells. Lapatinib was an even more potent inhibitor of cholangiocarcinoma cell growth and inducer of apoptosis than either tryphostin when tested in vitro against the respective rat and human cholangiocarcinoma cell lines, regardless of differences in their levels of ErbB1 or ErbB2 protein expression and/or mechanism of activation. This correlated with lapatinib inducing a prominent inhibition of both ErbB1 and ErbB2 signaling, activation of caspase-3, and suppression of cyclin D1. Moreover, lapatinib treatment produced a significant suppression of intrahepatic cholangiocarcinoma growth when administered early to rats but was without effect in inhibiting liver tumor growth and progression in rats with more advanced liver tumors. Overall, our findings support the concept that simultaneous targeting of ErbB1 and ErbB2 could be potentially useful as a neo-adjuvant therapy of intrahepatic cholangiocarcinoma.

37 Tumor-Specific Apoptosis Caused by Blockade of the ERBB3 Pseudo-Kinase

David W. Threadgill, Daekee Lee

Lineberger Cancer Center, University of North Carolina, Chapel Hill, NC; Department of Genetics, North Carolina State University, Raleigh, NC

Pharmacologic blockade of EGFR or the closely related receptor ERBB2 has modest efficacy against colorectal cancers in the clinic. Although the upregulation of ERBB3, a pseudo-kinase member of the EGFR/ERBB family, is known to contribute to EGFR-inhibitor resistance in other cancers, its functions in normal and malignant intestinal epithelium have not been defined. We show that the intestinal epithelium of mice with intestine-specific genetic ablation of Erbb3 exhibits no cytological abnormalities but does exhibit loss of expression of ERBB4 and sensitivity to intestinal damage. By contrast, intestine-specific Erbb3 ablation results in almost complete absence of intestinal tumors in the ApcMin mouse model of colon cancer. Unlike non-transformed epithelium lacking ERBB3, intestinal tumors lacking ERBB3 had reduced PI3K/AKT signaling, which led to attenuation of tumorigenesis via a tumor-specific increase in capase 3-mediated apoptosis. Consistent with the mouse data, which suggests that ERBB3-ERBB4 heterodimers contribute to colon cancer survival, loss of ERBB3 in a KRAS mutant human colon cancer cell line resistant to EGFR inhibition was associated with loss of ERBB4 expression, and siRNA knockdown of either ERBB3 or ERBB4 resulted in elevated levels of apoptosis. These results indicate that the ERBB3 pseudo-kinase has essential roles in supporting intestinal tumorigenesis and suggest that ERBB3 is a promising target for the treatment of KRAS mutant colorectal cancers.

38 Determinants of Tumor Sensitivity to EGFR-Targeted Antibodies

Louis M. Weiner[1], Sandra A. Jablonski[1], Yong Tang[1], Ilya Serebriiskii[2], Igor Astsaturov[2], Erica Golemis[2]

[1]Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC; [2]Fox Chase Cancer Center, Philadelphia, PA

This project has focused on understanding the critical structural determinants of anti-tumor antibodies that promote tumor-specific, antibody-dependent cellular cytotoxicity (ADCC), defining the antibody structural features and treatment strategies that maximize ADCC and promote the induction of adaptive immunity. Recently, our focus has shifted to analyzing the tumor-intrinsic factors that sensitize tumor cells to antibody therapy. We hypothesized that siRNA screening focused on genes functionally linked to the EGFR signaling pathway would identify tumor-intrinsic genes that regulate the tumor cell response to antigen engagement and ADCC promotion by monoclonal antibodies. To test this hypothesis, we have developed and applied a customized 638-element siRNA library containing genes known to functionally interact with EGFR (the EGFR functional "interactome"). Using this library, we identified a restricted number of genes whose knockdown selectively alters tumor cell viability in the presence of panitumumab and other EGFR inhibitors. We have identified "clusters" of genes known to act together in discrete subpathways, which we predict will be important for regulation of EGFR-family-directed signaling inhibition and ADCC. This project will dissect immunologic and signaling mechanism contributions to antibody efficacy and then identify efficacy-sensitizing genes for signaling and ADCC. Specific Aim 1 is to determine the roles of signaling inhibition and ADCC in mediating the efficacy of EGFR pathway-directed monoclonal antibodies. Specific Aim 2 is to define the elements of the EGFR interactome that modify target cell death in response to antibody engagement using cell culture-based functional tests to confirm the mechanisms by which siRNA depletion enhances tumor cell killing and in vivo models. In vitro and in vivo validation studies will identify new antibody-based therapy combinations. These studies will also identify those genes that regulate cellular sensitivity to EGFR inhibition and ADCC sensitivity. Specific Aim 3 is to determine the influence of mutations in critical signaling genes on EGFR antibody-targeted cytotoxicity. The completion of these aims will yield an improved understanding of the critical mechanisms that underlie successful antibody therapy and will form the basis for future monoclonal antibody treatment strategies.

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39 Role of Bromodomain 4 (Brd4) in Breast Cancer Progression and Metastasis

Jude Alsarraj[1], Nigel Crawford[1], Renard Walker[1], Luanne Lukes[1], Keiko Ozato[2], Kent Hunter[1]

[1]National Cancer Institute, National Institutes of Health, Bethesda, MD; [2]National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD

Metastasis is a complex process that remains a major problem in the management of cancer because most cancer deaths are attributed to a disseminated disease rather than the primary tumor. It is still unclear whether metastasis is driven primarily by somatic mutations within the tumor or subject to functional variations at the level of the whole organism. Understanding this component may be critically important to the early identification of patients at risk for metastasis and lead to a better prognosis and treatment. Constitutional genetic polymorphism has been associated with cancer risk and metastatic progression. Using the polyoma middle-T transgenic mouse mammary tumor model, we demonstrated that the genetic background upon which a tumor arose influenced the ability of the tumor to metastasize to the lung. Quantitative trait genetic mapping revealed the presence of a metastasis efficiency locus "Mtes1" on proximal mouse chromosome 19. Further analyses identified the signal transduction gene Sipa1 as a probable candidate for the Mtes1 locus. A polymorphism within SIPA1 was shown to positively correlate with the presence of metastases in breast cancer patients. Sipa1 interacts with Brd4, which is involved in cell growth regulation and is known to interact with acetylated chromatin. By using a highly metastatic mouse mammary tumor cell line Mvt-1 and stably expressing Brd4 in these cells, we found that Brd4 ectopic expression reduces cell invasion and migration in vitro. Subcutaneous implantation of these cells into mice showed that Brd4 activation reduces tumor growth and metastatic capacity. Gene expression comparisons between Brd4-expressing cells and control cells showed that genes previously associated with poor outcome in human breast cancers were differentially regulated and that Brd4-ectopic gene expression profile was highly predictive of outcome in human breast cancer datasets. Brd4 contains functional domains including two bromodomains, one of which is required for binding Sipa1, and a C-terminus containing a region required for interaction with the transcription elongation factor P-TEFb. We found that deletion of these domains changes the ability of Brd4 to affect tumor growth and metastasis. When these domains were deleted, Brd4 activation was associated with poor prognosis in human breast cancer datasets. Further experiments are in progress to understand the mechanisms in which Brd4 and its deletion mutants impact metastasis susceptibility.

40 Differential Expression of MicroRNAs in Endometrial Cancer-Activated Fibroblasts

Olga Aprelikova[1], Xiang Yu[1], John Palla[1], Simone John[1], Ming Yi[2], Robert Stephens[2], John Risinger[3], Amir Jazaeri[4], John Niederhuber[1]

[1]National Cancer Institute, Bethesda, MD; [2]National Cancer Institute, Frederick, MD; [3]Michigan State University, Grand Rapids; [4]University of Virginia, Charlottesville

The predominant component of tumor stroma consists of cancer-activated fibroblasts (CAFs). CAFs secrete growth factors and extracellular metalloproteinases that stimulate tumor growth and metastasis. The mechanism of CAF origination and re-programming remains unclear. We set out to identify the factors that may be involved in CAF reprogramming, such as microRNAs or transcription factors. We established matched pairs of fibroblast cell cultures from normal endometrium or endometrial cancer and analyzed differential expression of microRNAs and mRNAs. We found 11 microRNAs differentially regulated between normal and cancer fibroblasts, with 7 microRNAs being upregulated and 4 downregulated in CAFs. Mir-31 was the most downregulated microRNA and had a statistically significant enrichment of the predicted target genes in the differentially expressed mRNAs, whose expression negatively correlated with miR-31. Moreover, overexpression of miR-31 in CAFs repressed their ability to stimulate tumor cell migration and invasion. Of all predicted targets of miR-31, we focused on the homeobox gene SATB2 that was induced on average five-fold in CAFs. This gene is a matrix attachment-dependent chromatin remodeling protein that also functions as a transcriptional activator. We show that SATB2 is a direct target of miR-31 in stromal fibroblasts that functions in the promotion of cell motility and invasion. Since the tumor microenvironment is considered a target for therapeutic intervention, the discovered regulatory pathway gives us better understanding of the role of cancer-activated fibroblasts in tumor growth and progression and provides putative targets to control tumor microenvironment.

41 Patterns of Gene Expression and Copy-Number Alterations in VHL Disease-Associated and Sporadic Clear Cell Carcinoma of the Kidney

Rameen Beroukhim[1,2], Jean-Philippe Brunet[3], Arianna Di Napoli[2], Kirsten D. Mertz[2], Apryle Seeley[2], Maira M. Pires[2], David Linhart[3], Robert A. Worrell[5], Holger Moch[6], Mark A. Rubin[7], William R. Sellers[1,2,4], Matthew Meyerson[1,2], W. Marston Linehan[5], William G. Kaelin, Jr.[1,2,8], **Sabina Signoretti**[1,2]

[1]Dana-Farber Cancer Institute, Harvard Medical School; [2]Brigham and Women's Hospital, Harvard Medical School; [3]Broad Institute of the Massachusetts Institute of Technology and Harvard University; [4]Novartis Institutes for BioMedical Research; [5]National Cancer Institute; [6]University Hospital, Zurich, Switzerland; [7]Weill Cornell Medical Center; [8]Howard Hughes Medical Institute

Recent insights into the role of the VHL tumor suppressor gene in hereditary and sporadic clear cell carcinoma of the kidney (ccRCC) have led to new treatments for patients with metastatic ccRCC, although virtually all patients eventually succumb to the disease. We performed an integrated, genome-wide analysis of copy-number changes and gene expression profiles in 90 tumors, including both sporadic and VHL disease-associated tumors, in hopes of identifying new therapeutic targets in ccRCC. We identified 14 regions of nonrandom copy-number change, including 7 regions of amplification (1q, 2q, 5q, 7q, 8q, 12p, and 20q) and 7 regions of deletion (1p, 3p, 4q, 6q, 8p, 9p, and 14q). An analysis aimed at identifying the relevant genes revealed VHL as one of three genes in the 3p deletion peak, CDKN2A and CDKN2B as the only genes in the 9p deletion peak, and MYC as the only gene in the 8q amplification peak. An integrated analysis to identify genes in amplification peaks that are consistently overexpressed among amplified samples confirmed MYC as a potential target of 8q amplification and identified candidate oncogenes in the other regions. A comparison of genomic profiles revealed that VHL disease-associated tumors are similar to a subgroup of sporadic tumors and, thus, more homogeneous overall. Sporadic tumors without evidence of biallelic VHL inactivation fell into two groups: one group with genomic profiles highly dissimilar to the majority of ccRCC, and a second group with genomic profiles that are much more similar to tumors with biallelic inactivation of VHL.

42 Inhibition of FGFR in Endometrial Cancer Cells Induces Cell Death Both In Vitro and In Vivo, Demonstrating Novel Oncogene Addiction Despite Constitutive Activation of PI3K/AKT Signaling

Sara Byron[1], Michael Gartside[1], Candice Wellens[1], Mary Ann Malon[2], Feng Gao[2], Matthew Powell[2], Paul Goodfellow[2], **Pamela Pollock**[1]

[1]The Translational Genomics Research Institute, Phoenix, AZ; [2]Siteman Cancer Center, Washington University School of Medicine/Barnes-Jewish Hospital, St. Louis, MO

Endometrial carcinoma is the most common gynecological malignancy in the United States. Although most women present with early disease confined to the uterus, the majority of persistent or recurrent tumors are refractory to current chemotherapies. We have identified activating mutations in FGFR2 in a total of 51 of 515 (10%) endometrioid endometrial cancers. Survival analyses demonstrate that FGFR2 status is significantly associated with reduced progression-free survival and overall survival in early-stage tumors, suggesting that FGFR2 status could be an independent prognostic biomarker. FGFR2 and KRAS mutations occurred in a mutually exclusive pattern, while FGFR2 mutation frequently occurs alongside PTEN mutations. Inhibition of FGFR2 by two independent shRNAs inhibited cell proliferation and induced cell death in the AN3CA cell line. Western blot analysis revealed that this induction of apoptosis following knockdown of FGFR2 correlated with inhibition of phospho-ERK and occurred in the presence of constitutively phosphorylated AKT. To verify that inhibition with a pan-FGFR inhibitor was a viable therapeutic option in this tumor type, a panel of endometrial cell lines was treated with the pan-FGFR inhibitor PD173074 (Calbiochem). The three cell lines with mutant FGFR2 (AN3CA, MFE296, MFE280) were 10–40 times more sensitive to inhibition with PD173074. Notably, the most sensitive line has loss-of-function mutations on both PTEN alleles, and Western blot data confirmed this cell death occurred in the presence of constitutive AKT signaling. These data suggest that endometrial cancer cells with activated FGFR2 demonstrate a novel form of oncogene addiction. FGFR inhibition with PD173074 significantly inhibited tumor growth in the AN3CA human endometrial tumor xenograft model (activated FGFR2) but not in the HEC1A xenograft model (FGFR2 wild type). Six of eight AN3CA tumors showed regression with a mean tumor shrinkage of 51%, and the remaining two tumors demonstrated tumor growth inhibition of 91% compared to the mean tumor weight of the control group ($p<0.001$). Ongoing basic studies are concentrating on elucidating the novel caspase-independent, mitochondrial-dependent, CHX-dependent mechanism of cell death in response to FGFR inhibition with PD173074, and ongoing translational studies involve validating several multi-target kinase inhibitors including TKI258 and Brivanib as clinically relevant FGFR inhibitors in endometrial cancer.

43 Profiling Serum Antibodies With Carbohydrate Antigen Arrays

Christopher Campbell, Oyindasola Oyelaran, Yalong Zhang, David Farnsworth, Jeffrey Gildersleeve

Laboratory of Medicinal Chemistry, National Cancer Institute

Similar to changes in the repertoire of serum antibodies following immunization or exposure to pathogens, the profile of serum antibodies can change during onset and progression of cancer and other diseases. These changes in serum antibodies may reflect expression of abnormal antigens on tumors, immunologic response to the tumor, or non-specific changes due to cancer treatment. Methods to detect and interpret patterns of antibody expression associated with cancer hold promise for improved diagnosis and treatment. In early stage disease, antibodies could provide diagnostic biomarkers or aid prognosis. During development of vaccines, methods for detecting shifts in antibody expression could assist early evaluation of immune response. Many strategies have been developed to detect antibodies and monitor changes in antibody levels. We focus on carbohydrate-binding antibodies, whose significance is often under-recognized in comparison to antibodies directed against protein antigens. Carbohydrate-binding antibodies are a potentially high-yield subclass for biomarker discovery since they reflect altered glycosylation ubiquitous in cancer. Our group has developed a carbohydrate antigen array for high-throughput evaluation of carbohydrate-protein interactions. The array contains 204 different neoglycoconjugates and glycoproteins spotted on a glass slide using a robotic microarrayer. We have previously used the array to evaluate the specificities of antibodies and lectins used routinely to monitor expression of carbohydrate tumor antigens. Currently, we are using the array to profile the repertoire of anti-carbohydrate antibodies in human serum to discover new cancer biomarkers and to evaluate antibody responses generated with cancer vaccines.

44 Drosophila Sin3a Directs Cancer-Like Invasion Through Multiple Pathways

Tirtha K. Das, Ross L. Cagan

Mount Sinai School of Medicine, New York

Cancer progression involves acquisition of unlimited proliferative potential, invasion, and intravasation into the circulatory system, extravasation, and metastasis into distant sites. Invasion and metastasis involve complex interactions between transformed and wild-type cells. Cell-culture-based studies are inadequate to understand these processes, and modeling these events in a whole-animal context provides a more realistic picture of cancer progression in vivo. Drug therapy studies are also more informative in whole animal models as the drug's effect on the tumor as well as animal viability can be simultaneously assessed. Finally, modeling cancer in a genetically sophisticated system like Drosophila melanogaster allows for unbiased genetic modifier studies that can lead to identification of novel genes/pathways that modulate cancer progression in vivo. Our lab has established a Drosophila model of Multiple Endocrine Neoplasia Type 2 (MEN2), a rare aggressive cancer of the thyroid that arises from activating mutations in the proto-oncogene Ret. We had previously established Csk, a negative regulator of Src signaling, to be an important regulator of oncogenic Ret. We had also shown the drug ZD6474 to be a potent suppressor of MEN2 phenotype in the Drosophila eye. Our genetic modifier screen had identified Sin3a locus to be a strong modifier of oncogenic Ret, and our current studies explore the role of Drosophila Sin3a—an ortholog of the Sin3 proteins that regulate gene expression through their role as histone deacetylase (HDACs). Chromatin remodeling proteins regulate a broad variety of cellular processes. When misregulated, they are thought to direct aspects of tumorigenesis, although their precise roles in situ remain poorly understood. We demonstrate that Drosophila Sin3a is an important mediator of oncogenic Ret, and we provide evidence that Sin3a acts downstream of Src kinase and requires Jnk pathway activity; reduction of Sin3a activity can direct metastasis-like behavior in epithelial cells that results in secondary tumors. Further, we demonstrate that chemical inhibitors of Src and Jnk are effective in suppressing Sin3a-dependent overgrowth and migration in the context of our whole animal models. Together, our data suggest a model in which receptor tyrosine kinases such as Ret direct transformation and metastasis through a Src-Jnk-Sin3a axis. Targeting these pathways shows promise for therapeutic intervention.

45 Integrated Analysis of Kinase Signaling Pathways in Melanoma Clinical Specimens

Michael Davies, Jeffrey E. Gershenwald

The University of Texas M. D. Anderson Cancer Center, Houston

The prevalence of activating mutations (i.e., BRAF, NRAS, KIT) in kinase signaling pathways in melanoma provides a strong rationale to test targeted therapies in this disease. However, successful development of such therapies will likely depend upon an improved understanding of the activation status of protein signaling networks. In order to analyze mutations and protein activation in clinical specimens, we developed mass-spectroscopy-based genotyping (MS-genotyping) and reverse-phase protein arrays (RPPA). Using MS-genotyping, we can test for 150 different point mutations using 200 ng of DNA from frozen or FFPE specimens. RPPA allows for quantitative analysis of 80 proteins, using 30 μg of protein from frozen specimens, and can analyze up to 1,054 samples concurrently. In a pilot study, we analyzed OCT-embedded frozen melanoma metastases (n=96). Tumor-enriched regions were isolated by H&E-guided macrodissection. Both DNA and proteins were isolated from samples from these regions. RPPA analysis demonstrated good reproducibility for identical samples analyzed in different regions of the RPPA slides (r=0.99), as well as for different samples isolated from the same tumor (r=0.85). Activated AKT (P-AKT) levels demonstrated expected positive and negative correlations with known substrates (P-GSK3α/ß) and negative regulators (PTEN), respectively; similar correlations were observed in a panel of cell lines. These results demonstrate that phosphoproteins of interest were maintained during sample preparation and that RPPA results are reproducible. An integrated analysis of mutations and AKT pathway activation demonstrated that tumors with BRAF mutations had higher levels of P-AKT Ser473 (p=0.01), P-AKT Thr308 (p=0.002), and P-GSK3α/ß (p=0.08) than tumors with NRAS mutations. Analysis of the individual tumors demonstrated that almost all tumors with markedly elevated P-AKT had low levels of PTEN; NRAS-mutant tumors all retained PTEN expression and had P-AKT levels similar to BRAF-mutant tumors with normal PTEN and tumors with no detectable mutation. Analysis of tumors from different distant metastatic sites revealed that brain metastases had higher levels of P-AKT and P-GSK3α/ß and lower levels of PTEN than lung or liver metastases. These results demonstrate the power of integrated molecular analyses, suggest the feasibility of MS-genotyping and RPPA analysis of clinical specimens, and have implications for the development of targeted therapies for melanoma.

46 Identifying Novel Molecular Targets for T-Cell Lymphomas Based on Cytogenetic Findings

Andrew L. Feldman, Ahmet Dogan, Stephen M. Ansell

Mayo Clinic, Rochester, MN

T-cell lymphomas (TCLs) are fatal in the majority of patients and are increasing in incidence in the United States. Most patients receive chemotherapy designed for B-cell lymphomas (CHOP), with limited effectiveness. Targeted therapies could improve outcomes, but few molecular targets in TCLs are known. Recurrent chromosomal translocations have identified therapeutic targets in other hematopoietic tumors (e.g., BCR/ABL) but are rare in TCLs. We hypothesized that even rare translocations may identify novel therapeutic targets, since the involved pathways might be activated in non-translocated tumors by other mechanisms. We studied two genes involved in TCL translocations: SYK and IRF4. SYK encodes a tyrosine kinase involved in immunoreceptor signaling but absent in most normal T cells. SYK/ITK translocations exist in 1% of TCLs, leading to SYK protein expression. IRF4 encodes a transcription factor involved in T-cell activation. We discovered novel translocations involving IRF4 in TCLs (less than 7%), involving the T-cell receptor-alpha(TRAα) gene or unknown partner(s). The presence of IRF4 translocations had clinical utility as a diagnostic biomarker. Though SYK and IRF4 translocations were rare, SYK and IRF4 proteins were expressed in 94% and 42% of TCLs, respectively. SYK was phosphorylated in TCL patient samples, suggesting an activated state. In vitro, SYK siRNAs inhibited proliferation in TCL cells expressing phosphorylated (but not non-phosphorylated) SYK. Silencing SYK also induced apoptosis. IRF4 siRNAs inhibited proliferation in IRF4-positive TCL cells and downregulated expression of the proto-oncogene product, Myc. This finding is reminiscent of recent data in multiple myeloma and suggests IRF4 may play a different role in TCLs than in normal T cells, where it mainly acts as a transcriptional repressor. Taken together, our data suggest SYK and IRF4 represent candidate therapeutic targets for the treatment of TCLs. A SYK inhibitor is now in clinical trial for TCLs; specific IRF4 inhibitors are under development. More broadly, these data provide evidence that the TCL genome holds additional clues to novel molecular targets, even when primary genetic events such as translocations exist only rarely. Thus, a more comprehensive approach to characterizing the TCL genome is warranted.

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47 Expression of Histone Deacetylases in Lymphoma: Implication for the Development of Selective Inhibitors

Annunziata Gloghini[1], Daniela Buglio[2], Noor M. Khaskhely[2], Georgios Georgakis[2], Robert Z. Orlowski[2], Sattva S. Neelapu[2], Antonino Carbone[1], **Anas Younes**[2]

[1]Department of Pathology, National Cancer Institute, Milan, Italy; [2]Department of Lymphoma and Myeloma, The University of Texas M. D. Anderson Cancer Center, Houston

Unselective histone deacetylase (HDAC) inhibitors are a promising novel therapy for lymphoid malignancies. However, these treatments remain empiric as the pattern of HDAC enzymes in different types of cancer, including lymphoid malignancies, remains unknown. We examined the expression of class I and class II HDACs in a panel of cell lines and tissue sections from primary lymphoid tumors. Class I enzymes were highly expressed in all cell lines and primary tumors studied, including the non-malignant reactive cells in the Hodgkin lymphoma (HL) microenvironment. The most frequently altered HDAC expression was HDAC6, as it was either weakly expressed or undetected in 9 of 14 (64%) lymphoid cell lines and in 83 of 89 (93%) primary lymphoma tissue specimens, including 50 of 52 (96%) cases of diffuse large B-cell lymphoma and 18 of 22 (82%) cases of classical HL. Cell lines that had low expression level of HDAC6 demonstrated aberrant expression of hyper-acetylated tubulin and were found to be more sensitive to the growth inhibitory effects of the class I HDAC inhibitor MGCD0103. Collectively, our data demonstrate that HDAC6 is rarely expressed in primary lymphoma cases, suggesting that it may not be an important therapeutic target in these lymphoid malignancies.

48 An Integrative Genomic Analysis of PIK3CA, PTEN, AKT, KRAS, and BRAF Mutations in Endometrial Cancer

Bryan T. Hennessy, Larissa Meyer, Russell Broaddus, Karen Lu

The University of Texas M. D. Anderson Cancer Center

Introduction: Phosphatidylinositol 3-kinase (PI3K)/AKT and RAS/RAF signaling pathway aberrations are common in cancer and particularly common in endometrial cancer. Our aim was to determine the frequency, co-occurrence, and clinical effects of PIK3CA, PTEN, AKT, KRAS, and BRAF mutations in a large cohort of endometrial cancers.

Methods: We applied mass spectroscopy-based and Sanger sequencing to 200 human endometrial cancers obtained from the Gynecologic Tumor Bank at M. D. Anderson Cancer Center.

Results: PIK3CA mutations were detected in 52 tumors (26%), KRAS mutations in 36 (18%), and AKT1 mutations in 3 (1.5%) endometrial tumors. No BRAF mutations were detected. PTEN sequencing is ongoing. The intra-genic distribution of PIK3CA mutations was as follows: exon 1 (13 tumors; 25%), exon 9 (26; 50%) and exon 20 (13; 25%). In contrast, in 117 of 547 (21%) breast cancers in which we previously detected PIK3CA mutations, the distribution of PIK3CA mutations was as follows: exon 1 (1; 1%), exon 9 (43; 37%), and exon 20 (73; 62%). Therefore, in endometrial cancer, we detected significantly more exon 1 mutations in PIK3CA and significantly fewer exon 20 mutations in PIK3CA as compared to breast cancer ($p<0.0001$ for both comparisons). A KRAS mutation was detected in 25, 1, 6, 4, and 0 endometrial tumors that were PIK3CA wild- type, PIK3CA exon 1-mutated, PIK3CA exon 9-mutated, PIK3CA exon 20-mutated, and AKT1-mutated, respectively. There was no significant association between KRAS mutations and the presence or absence of PIK3CA mutations in endometrial cancer. Collection and analysis of clinical data is ongoing.

Conclusion: PIK3CA and KRAS mutations are common in endometrial cancer. In contrast to breast cancer, exon 1 mutations in PIK3CA are common in endometrial cancer. PIK3CA and KRAS mutations are not mutually exclusive in endometrial cancer.

49 BRCA Status in Ovarian Tumors

Bryan T. Hennessy[1], Kirsten Timms[2], Mark S. Carey[1], Jerry Lanchbury[2], Russell Broaddus[1], Karen Lu[1], Gordon B. Mills[1]
[1]The University of Texas M. D. Anderson Cancer Center; [2]Myriad Genetic Laboratories

Background: The rate of germline BRCA1 and BRCA2 mutations combined in normal DNA derived from sporadic ovarian cancer patients is approximately 13%. Trials of PARP inhibitors are underway in ovarian cancer patients with germline BRCA1/2 mutations. However, a large cohort of ovarian tumor tissues has not been studied to determine the frequency of BRCA deficiency due to mutations and other aberrations.

Methods: BRCA1 and 2 were sequenced in 235 high-grade ovarian cancers and 38 ovarian cancer cell lines. In 112 tumors, we also performed gene expression arrays and copy number arrays with ultradense tiling of probes throughout the BRCA genes.

Results: In BRCA1, there were 31 (13.2%) mutations: 23 known deleterious mutations, 1 suspected deleterious mutation, 3 novel indels, 3 novel nonsense mutations, and 1 novel missense mutation. For BRCA2, 178 samples have been sequenced and 12 mutations (6.7%) were detected: 8 known deleterious mutations, 1 suspected deleterious mutation, and 3 novel indels. Only three BRCA1 mutations were detected in two cell lines: two known deleterious and one novel 29 base pair deletion. One cell line thus appears to have both a germline and a somatic mutation. BRCA mutation status was associated with a trend to improved progression-free survival (PFS) in univariate analysis (p=0.17). However, BRCA deficiency—defined by BRCA1/2 gene expression loss (four tumors) and homozygous deletion (one tumor in addition to mutations)—was associated with improved PFS in univariate (p=0.04) and multivariate (p=0.03) analyses.

Conclusions: In both BRCA1 and BRCA2 genes, almost 25% of mutations in tumor tissue are novel mutations that have not been previously seen in germline DNA. We are sequencing corresponding germline DNA at present to determine whether these novel mutations are somatic. Direct analysis of high-grade ovarian tumor tissue is likely to expand the number of women with BRCA-deficient tumors beyond what is detectable by germline sequencing.

50 HMGA1: A New Biomarker and Therapeutic Target in Pancreatic Cancer

Hiromichi Ito, Siong Liau, Edward E. Whang
Brigham and Women's Hospital, Harvard Medical School, Boston, MA

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States. Most patients present with surgically incurable disease, and currently available chemotherapeutic agents have only a modest impact on survival. As such, there is a need for new therapeutic and diagnostic strategies. The high mobility group AT-Hook 1 gene HMGA1, which is located on chromosomal locus 6p21, encodes 2 HMGA1 splice variants (HMGA1a and HMGA1b). These HMGA1 proteins are architectural transcription factors that regulate gene expression. They are overexpressed in a range of human cancers, including pancreatic cancer. We hypothesized that HMGA1 is a candidate biomarker and therapeutic target in pancreatic adenocarcinoma. HMGA1 overexpression was induced in MiaPaCa2 human pancreatic cancer cells, which inherently express low levels of HMGA1. RNA interference was used to suppress HMGA1 expression in PANC-1 human pancreatic cancer cells, which inherently express high levels of HMGA1. Forced HMGA1 overexpression was associated with increased cellular invasive potential, increased resistance to anoikis, and increased chemoresistance to gemcitabine. Conversely, HMGA1 silencing was associated with decreased cellular invasive potential, decreased resistance to anoikis, and decreased chemoresistance to gemcitabine. In preclinical studies, BXPC-3 human pancreatic cancer cells in which HMGA1 had been silenced through stable lentiviral vector-mediated RNA interference or control BXPC-3 cells transduced with lentivirus carrying nontargeting shRNA were implanted into nude mice. Both groups of mice were treated with a 6-week course of gemcitabine. HMGA1 silencing increased the antitumor efficacy of gemcitabine in this model. Finally, immunohistochemical analysis of HMGA1 expression in pancreatic adenocarcinoma specimens from 89 consecutive patients having undergone resection of pancreatic cancer at our institution was performed. Tumoral HMGA1 expression was detected in 93% of patients with pancreatic adenocarcinoma. Patients with HMGA1-negative cancers had a significantly longer median survival than those with HMGA1-positive cancers. Tumoral HMGA1 expression status was an independent predictor of survival in multivariate analysis that included conventional pathological parameters, such as T stage, N stage, tumor differentiation, and lymphovascular/ perineural invasion status. In summary, HMGA1 shows promise as both a biomarker and a therapeutic target in pancreatic cancer.

51 Field Change in Bladder Carcinogenesis: The Concept of Forerunner Genes

Tadeusz Majewski, Sangkyou Lee, Shizhen Zhang, Sooyong Lee, Jolanta Bondaruk, Woonbok Chung, Jean-Pierre Issa, Menashe Bar-Eli, Keith Baggerly, **Bogdan Czerniak**

The University of Texas M. D. Anderson Cancer Center, Houston

Analysis of genomic imbalances can guide us to those chromosomal regions that contain genes playing a role in tumor development. In sporadic epithelial cancers that develop from microscopically recognizable in situ conditions, the early events maybe deduced from the geographic relationship between genomic imbalance and precursor in situ conditions. Using this approach, we matched the clonal allelic losses in distinct chromosomal regions to specific phases of bladder neoplasia and produced a detailed genetic map of human bladder cancer development. These analyses revealed three major waves of genetic changes associated with growth advantages of successive clones and reflecting a stepwise conversion of normal urothelial cells into cancer cells. The genetic changes mapped to six regions at 3q22–q24, 5q22–q31, 9q21–q22, 10q26, 13q14, and 17p13, which may be critical for the development of bladder cancer. In addition, we performed high-resolution mapping using single nucleotide polymorphism markers within one region on chromosome 13q14, containing the model tumor suppressor gene RB1, and defined a minimal deleted region associated with clonal expansion of in situ neoplasia. These analyses provided new insights on the involvement of several noncoding sequences mapping to the region and identified novel target genes, termed forerunner (FR) genes, involved in early phases of cancer development. The initial functional studies focused on the two nearest candidate FR genes flanking RB1. These were ITM2B, which encodes a mitochondrial membrane protein with a BH3 domain, and CHC1L, which encodes a GEF protein for the ras-related GTPase. Surprisingly, a third candidate FR gene, P2RY5, was actually located within intron 17 of RB1 and encodes a G-protein-coupled receptor. ITM2B and P2RY5 modulated cell survival and were silenced by methylation or point mutations, respectively, and thus by functional loss may contribute to the growth advantage of neoplasia. We also showed that homozygous inactivation of P2RY5 was antecedent to the loss of RB1 during tumor development and that nucleotide substitutions in P2RY5 represent a cancer predisposing factor. Our recent studies of another candidate FR gene, ARL11 encoding ADP-ribosylation factor-like tumor suppressor protein 1, have identified an unexpected link between the recessive events in the 13q14 region and upregulation of one of the most important oncogenic pathways, namely the ras signaling.

52 Induced Chromosomal Proximity and the Genesis of Gene Fusions in Prostate Cancer

Ram-Shankar Mani[1,2], Scott A. Tomlins[1,2], Kaitlin Callahan[1,2], Aparna Ghosh[1,2], Sooryanarayana Varambally[1,2,3], Mukesh K. Nyati[3,4], Nallasivam Palanisamy[1,2], Arul M. Chinnaiyan[1,2,3,5,6]

[1]Michigan Center for Translational Pathology; [2]Department of Pathology, [3]Comprehensive Cancer Center, [4]Department of Radiation Oncology, [5]Department of Urology, University of Michigan Medical School, Ann Arbor; [6]Howard Hughes Medical Institute

Gene fusions have a critical role in cancer progression. Mechanisms associated with the genesis and cell type specificity of these fusions are not well understood. A prototypical gene fusion, TMPRSS2-ERG, involves the 5'-untranslated region of androgen-regulated gene TMPRSS2 with the ERG gene and is the most common, found in approximately 50% of prostate cancers. We demonstrate that androgen signaling induces chromosomal proximity between TMPRSS2 and ERG loci and facilitates the formation of the TMPRSS2-ERG gene fusion when subjected to an agent that causes DNA double strand breaks. These results provide a conceptual framework for the genesis of gene fusions and may provide suggestions as to the general etiology of human prostate cancer.

53 Wnt Signaling, A Novel Target for Ovarian Cancer Therapeutics

Jasmine Nicodemus[1], Keri Soring[1], Hong Wu[1], Michael J. Birrer[2], Denise C. Connolly[1], Andrew Godwin[1], Paul Cairns[1], **Rugang Zhang**[1]

[1]Fox Chase Cancer Center, Philadelphia, PA; [2]Massachusetts General Hospital Cancer Center, Harvard Medical School, Boston

Ovarian cancer is the most lethal gynecological cancer in the United States. There is an urgent need to identify novel therapeutic targets for treatment of the cancer. The canonical Wnt signaling plays an important role in maintaining proliferation potential of tissue stem/progenitor cells and is often activated in human cancers. However, the role of Wnt signaling in ovarian cancer is not fully understood. We found that canonical Wnt signaling is active in human ovarian cancer cells, and inhibition of canonical Wnt signaling inhibits the growth of human ovarian cancer cells. A non-canonical Wnt ligand, Wnt5a, is inevitably downregulated in human ovarian cancer cell lines and in primary human ovarian tumors compared to primary human ovarian surface epithelial (HOSE) cells. In addition, canonical Wnt1 is expressed in both primary HOSE cells and human ovarian cancer cell lines. Promoter DNA methylation contributes to the downregulation of Wnt5a in human ovarian cancer cells. Importantly, low level of Wnt5a expression predicts poor overall survival of ovarian cancer patients. Significantly, restoration of Wnt5a expression or knockdown of Wnt1 expression inhibits canonical Wnt signaling and represses both anchorage-dependent and -independent growth of human ovarian cancer cells. Conversely, knockdown of Wnt5a expression activates canonical Wnt signaling in primary HOSE cells. We conclude that Wnt5a antagonizes canonical Wnt1 signaling in primary HOSE cells and downregulation of Wnt5a activates Wnt1 signaling in human ovarian cancer cells. Our results suggest that Wnt signaling may be a novel target for therapeutics of human ovarian cancer.

54 Pituitary Adenylyl Cyclase Activating Peptide (PACAP) Regulates Medulloblastoma Pathogenesis in ptc1 Mutant Mice and Counter-Regulates Multiple Hedgehog Target Genes

Pawel Niewiadomski, Joseph Cohen, Jennifer Kong, Daniel Resnick, Annie Zhujiang, Linda M. Liau, **James A. Waschek**

University of California, Los Angeles

In flies and vertebrates, Hedgehog proteins and cAMP-dependent protein kinase A (PKA) generally play opposing roles in developmental patterning events. Humans and mice heterozygous for mutations in the Sonic hedgehog (SHH) receptor gene patched-1 (ptc1) have an increased incidence of certain types of cancer, including medulloblastoma (MB), a highly aggressive tumor of young adults and children. Receptors for pituitary adenylyl cyclase activating peptide (PACAP) and SHH are coexpressed in the germinal centers that are thought to give rise to MB. We hypothesized that loss of PACAP might synergize with the ptc1 mutation to increase susceptibility to these tumors. Mutation of a single copy of PACAP increased MB incidence approximate 2.5-fold, to 66%. Moreover, tumors in ptc1+/– mice could be detected in vivo by positron-emission tomography (PET) at least 6 weeks before the onset of symptoms. To identify novel PACAP-sensitive target genes that might be involved in MB, cultures of mouse cerebellar granule precursor—the cells thought to give rise to MB—were treated with SHH, PACAP, or the combination. The expression of the great majority of genes induced by SHH were blocked by PACAP, including several novel SHH target genes. These genes were found to be expressed in the cerebellum at highest levels during postnatal development and were also expressed in mouse MB tumors. The results implicate PACAP as a physiological PKA activating factor that regulates the incidence and/or growth of hedgehog pathway-associated medulloblastoma tumors in mice and identify a set of novel genes that are cross-regulated by these signaling pathways.

55 Selective Expression of CD44, A Putative Prostate Cancer Stem Cell Marker, In Neuroendocrine Tumor Cells of Human Prostate Cancer

Ganesh S. Palapattu[1], Chengyu Wu[1], Christopher R. Silvers[1], Heather B. Martin[1], Karin Williams[1], Linda Salamone[1], Timothy Bushnell[1], Li-Shan Huang[1], Qi Yang[1], **Jiaoti Huang**[2]

[1]University of Rochester; [2]University of California, Los Angeles

Hormonal therapy is effective for the treatment of advanced prostate cancer (PC) initially, but the disease often recurs and becomes hormone-refractory. The mechanism for the development of hormone-refractory PC is unclear, and many hypotheses have been proposed. One hypothesis states that a subpopulation of cancer cells, the so-called cancer stem cells (CSCs), survives hormonal therapy and leads to tumor recurrence. Much effort has been devoted to the identification of the CSCs. Among the putative cell surface CSC markers, CD44 expression has been shown to identify tumor cells with CSC features. PC contains secretory-type epithelial cells and a minor subpopulation of neuroendocrine (NE) cells. NE cells compose approximately 1% of the tumor cells. They do not express luminal differentiation markers androgen receptor and PSA and are normally quiescent. Their number increases in hormonally treated and hormone-refractory PCs. These features have all been associated with CSCs. Therefore, we studied the expression of CD44 in human PC and its relationship to NE tumor cells. Immunohistochemistry and immunofluorescence studies were performed to study CD44 expression in PC cell lines, single cells from fresh PC tissue, and archival tissue sections of PC. We then determined the relationship of CD44 expression with NE tumor cells. In human PC cell lines, expression of CD44 is associated with cells of NE phenotype. In human PC tissues, NE tumor cells are virtually all positive for CD44, and CD44-positive cells, excluding lymphocytes, are all NE tumor cells. Therefore, our study has demonstrated that the putative CSC marker CD44 is selectively expressed in NE tumor cells of PC. This interesting finding, in combination with other known features of NE cells, further supports the potential significance of NE tumor cells in the development of therapy resistance and tumor recurrence after hormonal therapy.

56 Combining an Integrative Genetic and Genomics Approach and Therapeutic Studies Identifies the NFkb Pathway as Crucial in BRAF Mutant Melanomas

Maurizia Dalla Palma[1], Michelle Korenblit[2], Keiran Smalley[3], Bin Li[1], Rachel Hammond[2], Patricia Van Belle[4], David Elder[4,5], Yan Wang[1], Meenhard Herlyn[6], Phyllis Gimotty[2,5], **Katherine L. Nathanson**[1,5]

Departments of [1]Medicine, Medical Genetics, [2]Epidemiology and Biostatistics, University of Pennsylvania, Philadelphia; [3]Moffit Cancer Center, Tampa, FL; [4]Pathology Department and [5]Abramson Cancer Center, University of Pennsylvania, Philadelphia; [6]Wistar Institute, Philadelphia, PA

No effective therapy for the treatment of advanced stage melanoma has been identified to date. However, targeted therapies against BRAF mutations are showing promise in clinical trials. Genetic and genomic analyses of melanomas are being used to stratify tumors with the eventual goal of determining the optimum treatment for each patient. We genotyped metastatic melanomas (27 cell lines, 22 paraffin, and 34 frozen tumors) for BRAF and NRAS mutations and used BAC array CGH to identify chromosomal aberrations. Using a 2-way ANOVA test, we demonstrated that BRAF mutational status was associated with a specific profile of DNA copy number aberrations. As compared to non-BRAF mutant melanoma, those with BRAF mutations contained significantly more frequent amplifications of large regions of chromosome 7, 20p12-11 and 10q24-25 deletion ($p<0.05$). Using a computational network analysis to discern molecular interactions within the genomic profile identified, we identified MAPK and NFkB signaling pathways as most predominant ($p<0.0002$). To confirm our findings, we evaluated NFkB p65 activation in melanoma cell lines. We demonstrated that BRAF mutant melanoma cell lines showed significantly higher levels of nuclear RelA/p65 activation than either NRAS mutant or both BRAF/NRAS wild-type cell lines, with almost no nuclear RelA/p65. We used pharmacological inhibitors to demonstrate utility of combining BRAF/MEK and IKK inhibition. MEK inhibitor treatment reduced cell growth, blocking the cell in G1 phase of the cell cycle, but did not increase apoptosis; IKK inhibitor caused a G2/M arrest and enhancement of apoptosis, particularly in melanoma cell lines carrying a BRAF mutation. In a two-dimensional culture, combined treatment with the two drugs was additive in BRAF-mutant, but not BRAF wild-type, melanoma cell lines, leading to simultaneous G1 and G2/M block and increased apoptosis. In three-dimensional collagen-implanted spheroid culture conditions, use of MEK and IKK inhibitors simultaneously reduced spheroid size, number, and ability to invade collagen, again solely in BRAF-mutant melanoma cell lines. Together, the two drugs cause apoptosis through mitochondrial mediated mechanisms with 40% loss of the mitochondrial potential in BRAF-mutant compared to wild-type melanoma cell lines. These data demonstrate integrative genetic approaches can be used to identify cooperating activated pathways that in turn suggest effective targeted therapy combinations for melanoma. Thus, we would suggest that NFκB inhibitors may be used in combination with MAPK pathway targeted therapy, specifically for the treatment of BRAF-mutant melanomas.

57 Targeting the mTOR Pathway for Treatment of Non-Small Cell Lung Cancer

Suresh S. Ramalingam, Taofeek K. Owonikoko, Shiyong Sun, Dong M. Shin, Haian Fu, Fadlo R. Khuri

Emory University, Winship Cancer Institute, Atlanta, GA

The mammalian target of rapamycin (mTOR) pathway plays a central role in regulating cell growth, proliferation, and survival. Approximately 70% of non-small cell lung cancer (NSCLC) tumors have activated mTOR signaling. In addition, the PI3/Akt signaling is dysregulated in a majority of NSCLC tumors. We are conducting two clinical trials to evaluate the anti-cancer effects of everolimus (RAD001), a novel inhibitor of mTOR. In the first study, patients with surgically resectable NSCLC (stages I, II, or III) are treated with 3 weeks of therapy with everolimus following a pretreatment tumor biopsy. On day 22, patients undergo surgical resection for the primary tumor, at which time tumor tissue is also harvested for biomarker analysis. PET scans are obtained at baseline and after 3 weeks of therapy with everolimus (before surgery). The primary endpoint is to evaluate molecular changes downstream and upstream of mTOR in the tumor with everolimus therapy by comparing the pretreatment biopsy specimen with the surgical specimen. A total of eight patients have been enrolled to date. Everolimus was well tolerated at a dose of 5 mg by mouth every day, and all patients underwent the planned surgical procedure. Metabolic response, defined by a reduction in the FDG uptake in the tumor was noted in nearly all patients. As anticipated, upregulation of AKT activity was noted in the tumors with everolimus therapy. Additional biomarkers are currently under evaluation, and accrual to the study continues. In the second study, the safety and efficacy of the combination of everolimus with docetaxel is being evaluated. Based on our preclinical data that demonstrated synergy between the two agents, we have completed a Phase I study in patients with advanced stage NSCLC. Twenty-four patients were enrolled to the study (median age: 62; female: 11; ECOG PS: 0 [6], 1 [17], 2 [1]; number of prior regimens: 1 [13], 2 [6], more than 3 [5]). The dose-limiting toxicities (DLT) were fever with grade 3/4 neutropenia, grade 3 fatigue, and mucositis. None of the six patients treated at the recommended Phase II dose of docetaxel 60 mg/m2 and everolimus 5 mg QD experienced DLT. The mean everolimus half-life in hours on days 1, 8, and 15 were 9.66, 12.6, and 14.8, respectively. The mean area under the concentration-time curve (AUC)-based accumulation factors for everolimus on day 8 and 15 were 1.78 and 1.88, respectively. Among 21 patients evaluable for response, 1 had a partial response, and 10 had disease stabilization. Based on the favorable tolerability profile and efficacy signal, we are now conducting a Phase II study for second line therapy of advanced NSCLC with this novel combination.

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58 Androgen Receptor Splice Variant Expression and Function in Prostate Cancer

Shihua Sun[1], Elahe Mostaghel[1,2], Ilsa Coleman[1], Kathy Haugk[3], Robert Vessella[1,3], Peter S. Nelson[1,3], **Stephen R. Plymate**[1,3]

[1]Department of Medicine, University of Washington, Seattle; [2]Department of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA; [3]Geriatric Research Education and Clinical Center Puget Sound VA Health Care System, Seattle, WA

In this study, we describe a new splice variant of the androgen receptor that was discovered from sequencing AR mRNA from 27 human prostate xenografts. Two of the xenografts had an AR splice variant that composed more than 90% of the total AR mRNA. Sequencing of this variant revealed a loss of exons 5, 6, and 7. Exon 8 continued after exon 4, but due to a frame shift, a stop codon limited exon 8 to the first 12 AA. The sequence was identical in the two xenografts, although they were from different patients. Examination of the primary tissue from which the xenografts were derived showed the AR variant (ARvar) to have been the major AR. The ARvar construct was made and expressed in AR-negative M12 cells. ARE luciferase reporter assays demonstrated the variant to be constitutively active at a higher level of activity than the full-length wtAR. Fluorescent microscopy showed the ARvar to be in the cell nucleus in the absence of androgens consistent with constitutive activity. When expressed in LnCaP cells such that cells expressed both the native receptor and ARvar, there was a physical interaction such that the cells were responsive to significantly lower levels of dihydrotestosterone (10–16M) than LnCaP cells without ARvar (10–12M) as measured by proliferation and luciferase reporter activity. When human prostate xenografts containing low, medium, or high levels of ARvar in relation to wtAR were injected subcutaneously into SCID mice and animals were castrated after tumors reached 200mm3, no response in tumor volume or PSA was seen, medium levels had a slowing of tumor growth and PSA increase, and tumors with low levels of ARvar 5, 6, 7 had a following castration. Finally examination of laser captured benign and malignant prostate epithelium from prostatectomy specimens revealed ARvar 5, 6, 7 in benign as well as malignant tissue; it was found more commonly in metastatic prostate tissue, but the variant could also be detected in prostate biopsies from young (35–45-year-old) men with no evidence of prostate cancer and normal PSA levels. These data show that (1) ARvar is frequently found in prostate cancer, (2) it functions independently but also enhances the response of the full length receptor to ligand, (3) the level of ARvar 5, 6, 7 may determine response to castration, and (4) ARvar 5, 6, 7 is expressed in normal epithelium but when present in malignant epithelium may be a poor prognostic factor for response to androgen ablation.

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59 GSK3-Beta as a Potential Therapeutic Target in Endometrial Cancer

Premal H. Thaker, Bobbie J. Rimel, Y. Yin, S. Arur, Tim Schedl, Liang Ma, Paul J. Goodfellow

Siteman Cancer Center, Washington University School of Medicine, St. Louis, MO

Endometrial cancer is the most common gynecologic malignancy. Effective non-toxic therapies for advanced or recurrent endometrial cancer are lacking. Recently, successful biologic therapies in other tumors have paved the way for exploring targeted therapies for endometrial cancer. Laboratory findings have proven mutational activation of the extracellular signal-regulated kinase (ERK) pathway is a frequent oncogenic event in endometrial cancers. ERK signaling is mediated by phosphorylation of substrate proteins, and to date few of the effector molecules have been identified and characterized. We have employed a 3-part functional genomics approach to identify novel evolutionary conserved ERK substrates that function in C. elegans germ cell development. One of the ERK substrates that inhibits ERK-dependent processes, glycogen synthase kinase 3-beta (GSK3b), was chosen for investigation as a potential therapeutic target. GSK3b's role in cancer biology has been well-established. GSK3b functions in canonical Wnt signaling, plays a critical role in NFkB signaling, and is important for cell proliferation/survival. To assess GSK3b in endometrial cancer cell proliferation, we inhibited GSK3b activity using either lithium chloride or AR-A014418 (inhibitor VIII) in endometrial cancer cell lines AN3CA, HEC1-A, ISHIKAWA, and SPEC-2 and in EM-E6/E7 TERT CS transformed normal endometrial cell line. Fifty um inhibitor was cytotoxic in all cancer cell lines but not in the transformed cell line. To determine the effects of inhibitor VIII on proliferation, we performed a time course experiment. Cell lines were treated with either DMSO (vehicle) or inhibitor VIII 24 hours after plating. Cell counts revealed that GSK3b inhibitor VIII inhibited cell proliferation as early as 24 hours post-treatment in all cancer cell lines, and the effect persisted for 72–96 hours. Mouse knockout studies demonstrated GSK3b is not required for cell survival/proliferation in normal cells; GSK3b null embryos can survive to midgestation, and a mutant embryonic fibroblast cell line has been established. The findings from our cancer cell line studies suggest a tumor-specific function for GSK3b in promoting cell growth and that the function can be blocked by GSK3b inhibition. We have initiated experiments to assess the effects GSK3b inhibitors have in an in vivo orthotopic model of endometrial cancer. Antibodies that recognize the ERK phosphorylated form of GSK3b are being evaluated in endometrial cancer cell lines and primary tumors to determine the relationship between ERK activation, GSK3b phosphorylation status, and clinico-pathologic features.

60 RNAi Identified Lethal Targets and Bortezomib Sensitizors in Myeloma

R.E. Tiedemann[1], Y.X. Zhu[1], J. Schmidt[1], C.X. Shi[1], H. Yin[2], Q. Que[2], S. Mousses[2], **A.K. Stewart**[1]

[1]Comprehensive Cancer Center, Mayo Clinic, Scottsdale, AZ; [2]Translational Genomics Institute, Scottsdale, AZ

Introduction: We have conducted systematic RNA interference (RNAi) lethality screening of the "druggable genome" in human myeloma cell lines (HMCL) to functionally generate a comprehensive map of molecular vulnerabilities in human myeloma tumor cells. Targets that modulate myeloma cell sensitivity to proteosome inhibition were simultaneously identified from a functional genomics approach.

Methods: KMS11 human myeloma cells were screened first with an 1,800-oligo small interfering RNA (siRNA) library targeting the kinome and subsequently with a 13,984-oligo library targeting the druggable genome (6,791 genes), in duplicate, using optimized conditions that resulted in greater than 95% transfection efficiency. Each gene was screened with at least two distinct oligos, in both the absence and presence of titrated bortezomib (IC10-IC50), using a single-siRNA-per-well format, testing more than 150,000 wells. Universally lethal and non-silencing siRNA were employed as controls. Viability was measured at 96h by ATP-dependent luminescence, normalized by B-score. Bortezomib chemo-sensitization was assessed by Bliss independence. The specificity (FDR) of lethal or bortezomib-modulating RNAi was evaluated by custom-developed RNAi concordancy statistical methods, and more than 300 candidate targets were validated in secondary and tertiary studies using four siRNA per gene. Validated targets were further examined in multiple cell lines and primary samples for expression and myeloma-specific vulnerability using viral RNAi techniques.

Results: Approximately 14% of kinome and 5.8% of druggable genome siRNA cause reductions in HMCL viability (greater than 3 standard deviations from non-silencing siRNA). Validated myeloma survival targets include 24 kinases and 39 other genes. Directly vulnerable kinases are concentrated within cytokine pathways (VEGFR, FGFR3, IGFR/IL6R signaling) or are involved in the regulation of cell cycle, apoptosis, or metabolism. Non-kinase vulnerabilities are centered on the proteosome, mitotic spindle, transcription, protein anabolism, and regulation of apoptosis. Bortezomib chemo-modulating targets include ER stress-transducing and cyclin-dependent kinases and genes of novel function. While many targets such as polo-like kinase 1 (PLK1) appear universally lethal, other targets such as G-protein receptor coupled kinase (GRK6) show specific cytotoxicity in myeloma.

Conclusion: We have identified approximately 60 critically vulnerable targets essential for proliferation or survival of myeloma cells and have additionally defined targets that are synergistic with bortezomib. Small molecule inhibitors are now being studied.

61 Activated Wound Responses in Normal Breast of Invasive Breast Cancer Patients

Melissa A. Troester

Lineberger Comprehensive Cancer Center and Department of Epidemiology, University of North Carolina at Chapel Hill

Commonalities between healing wounds and invasive cancers have led to the concept of cancer as an "overhealing wound" or a "wound that does not heal." This analogy between cancer and wound response was originally based on cellular composition changes and gross histological observations; however, more recent work suggests that an activated wound response is evident in tumor gene expression. The activated gene expression signature is associated with tumor progression and predicts outcome in breast cancer patients. While wound response gene expression is now well established in tumors, the activation of wound responses in the normal tissue adjacent to cancer has not been examined. Using more than 100 normal breast tissue samples from women undergoing reduction mammoplasty, excisional biopsy, or mastectomy, we used microarrays to characterize gene expression alterations associated with breast cancer. Our data show that wound responses are also activated in the normal microenvironment adjacent to the tumor. A signature of wound response in the normal microenvironment has greater than 95% accuracy in distinguishing patients with disease from those without disease based solely on gene expression in their normal tissue. Altered gene expression in the normal tissue of cancer patients has translational implications. Given the higher rate of recurrence among women undergoing breast-conserving therapy compared to women undergoing mastectomy, characterization of gene expression alterations in the normal microenvironment could lead to biomarkers for recurrence risk and could provide biologic evidence to support decisions on surgical margin width.

62 Overexpression of Smoothened Activates the Sonic Hedgehog Signaling Pathway in Pancreatic Cancer-Associated Fibroblasts

Kimberly Walter[1], Noriyuki Omura[1], Seung-Mo Hong[1], Margaret Griffith[1], Audrey Vincent[1], Michael Borges[1], **Michael Goggins**[1,2,3]

Departments of [1]Pathology, [2]Medicine, and [3]Oncology, The Sol Goldman Pancreatic Research Center, Johns Hopkins Medical Institutions, Baltimore, MD

Although the Hedgehog signaling pathway is aberrantly expressed in pancreatic cancers, accumulating evidence from mouse models suggests that the Sonic Hedgehog ligand (Shh) is not active in tumor epithelial cells and instead requires a paracrine signaling mechanism. However, it is not known if this paracrine mechanism of Hedgehog signaling can be extended to human stroma. We performed gene expression profiling of human pancreatic cancer-associated fibroblasts (CAFs) and non-neoplastic pancreatic fibroblasts to identify differentially expressed genes in CAFs. Among the genes upregulated in cancer-associated fibroblasts relative to control fibroblasts was the Hedgehog receptor Smoothened (SMO). We find that CAFs expressing SMO can transduce the Shh signal to activate Gli1 expression, and siRNA knockdown of SMO blocks the induction of Gli1 in these cells. We further find overexpression of SMO in stromal fibroblasts of human primary pancreatic adenocarcinomas compared to normal pancreatic fibroblasts. These findings implicate overexpression of SMO as a mechanism for the activation of Hedgehog signaling in human pancreatic CAFs and suggest that stromal cells may be a therapeutic target for SMO antagonists in pancreatic cancer.

63 Targeting LKB1 Signaling in Lung Cancer

Wei Zhou[1], Diansheng Zhong[1], Li Xiong[2], Xiuju Liu[1], Adam Marcus[1], Jing Chen[1], Shi-Yong Sun[1], Fadlo R. Khuri[1]

Departments of [1]Hematology and Medical Oncology, [2]Mathematics and Computer Science, [3]Pathology and Laboratory Medicine, and [4]Human Genetics, Emory University, Atlanta, GA

LKB1 is frequently inactivated in non-small cell lung cancer because it is involved in the regulation of cell polarity and mTOR signaling. Recent animal model studies also indicated that the loss of LKB1 facilitates tumor metastasis, suggesting that LKB1 inactivation may be more prevalent in lung metastasis. Interestingly, LKB1 has another function that promotes cell survival: it phosphorylates and activates AMPK in times of reduced energy availability, thus representing a metabolic checkpoint that inhibits a variety of ATP-consuming biosynthetic processes such as protein, fatty-acid, and cholesterol synthesis, while stimulating ATP-generating catabolic pathways including fatty acid-oxidation and autophagy. Such a metabolic point promotes cell survival in times of energy stress, and LKB1-deficient mouse embryonic fibroblasts cells are hypersensitive to apoptosis induced by energy stress. Based on these experimental observations, we evaluated the ability of 2-deoxyglucose, a glycolytic inhibitor, to induce cell killing in LKB1-deficient non-small cell lung cancers. Surprisingly, we discovered that in addition to the inhibition of glycolysis, 2-deoxyglucose also activates Akt and ERK signaling through IGF1R. Furthermore, targeted inhibition of either PI3K/AKT or MEK/ERK alone was not sufficient to substantially negate the survival effect that 2-deoxyglucose induced. In contrast, a synergistic effect on growth inhibition was observed when 2-deoxyglucose was combined with an IGF1R inhibitor. Therefore, we propose that the combination of 2-deoxyglucose and IGF1R inhibitors may be used as a novel therapy for the treatment for lung cancers with LKB1 inactivation.

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64 Methylation-Based Detection of Kidney Cancer

Paul Cairns, Robert G. Uzzo

Fox Chase Cancer Center, Philadelphia, PA

Kidney cancer confined by the renal capsule can be surgically cured in the majority of cases, whereas the prognosis for patients with advanced disease at presentation remains poor. Novel strategies for early detection are therefore needed. Kidney tumors are heterogeneous in their histology, genetics, and clinical behavior. We have found that promoter hypermethylation of tumor suppressor genes is common, can occur relatively early, may disrupt critical pathways, and, thus, likely plays a critical role in kidney tumorigenesis. We have also demonstrated sensitive and specific early detection of renal cancer in urine using a hypermethylated gene panel. We have generated and communicated to the field metrics for validation of methylation-based detection of cancer. To advance our goals, we have recently completed a comprehensive gene methylation profile to determine the timing of methylation in early stage, curable renal cancer and identify the optimal methylated genes for inclusion in panel for early detection. Our study of urines obtained at followup of patients with no clinical evidence of disease several weeks or months after undergoing nephrectomy for organ-confined disease further supports the specificity of gene methylation for the presence of cancer and, importantly, may prove to be a useful procedure for molecular monitoring of renal cancer. We have performed a demethylating drug-based global epigenetic reactivation in renal carcinoma cells and thereby generated the first pass of the renal cancer cell methylome. Novel candidate tumor suppressor genes identified by this screen are profiled for methylation status in primary early stage renal tumors to determine utility for early detection and differential diagnosis of kidney cancer.

65 NGAL from Pancreatitis to Pancreatic Cancer: Diagnostic and Prognostic Implications in Patients

Subhankar Chakraborty[1], Sukhwinder Kaur[1], Nicholas Moniaux[1], Venkata Muddana[3], Uwe A. Wittel[4], Kavita Mallya[1], Georgios I. Papachristou[3], David Whitcomb[3], Aaron R. Sasson[2], Randall E. Brand[3], Michael A. Hollingsworth[2], **Surinder K. Batra**[1,2]

[1]Department of Biochemistry and Molecular Biology, [2]Eppley Institute for Cancer Research, University of Nebraska Medical Center, Omaha; [3]Division of Gastroenterology, University of Pittsburgh Medical Center, Pittsburgh, PA; [4]Universitätsklinik Freiburg, Freiburg, Germany

Pancreatic cancer (PC) is a highly lethal malignancy with a dismal 5-year survival. Neutrophil gelatinase-associated lipocalin (NGAL) was reported to be upregulated nearly 27-fold in PC cells compared to normal pancreatic ductal cells by microarray analysis. The objective of our study was to examine whether NGAL could be used to identify foci of pancreatic dysplasia in tissue sections and examine the role of serum NGAL as a marker in pancreatitis and PC. NGAL expression was almost undetectable in normal pancreas, while being strongly upregulated in PC tissues and moderately in pancreatitis. While both well and moderately differentiated PC were positive, areas of poorly differentiated adenocarcinoma were uniformly negative. Importantly, NGAL was detected in the pancreatic tissues as early as the PanIN-1 stage, suggesting that it could be an early marker of dysplastic change. Plasma NGAL levels were significantly higher in PC and pancreatitis patients compared to healthy controls. Interestingly, NGAL levels were significantly higher in non-metastatic PC compared to metastatic PC patients. In a mouse model of acute pancreatitis, significantly higher levels of NGAL were observed in mice that developed features of severe pancreatitis (SAP) compared to those that developed mild pancreatitis (MAP). Interestingly, in mice with SAP, the elevation in NGAL was observed within 6 hours of induction and reached a peak after 24 hours. In AP patients, mean (±SE) serum NGAL was significantly higher in SAP (634±139 ng/ml) compared to MAP cases (84.7±7 ng/ml, p=0.0001) for samples collected within 5 days of onset of symptoms. Upon subanalysis, the difference between the two groups was significant in serum samples collected within 48 hours but not at 72, 96, or 120 hours. NGAL was 100%, 96%, 97%, and 84% specific and 100%, 87.5%, 92%, and 94% sensitive in distinguishing SAP from MAP at 2, 3, 4, and 5 days after the onset of symptoms. Significantly, NGAL levels were higher in SAP cases complicated by multi-organ failure and appeared to correlate with a fatal outcome. In conclusion, NGAL expression could be potentially useful to identify early dysplastic changes in the pancreas. Further, while serum NGAL measurement does not discriminate well between pancreatitis and PC, it is highly elevated in plasma within the first 24 hours of onset of SAP. High NGAL levels appear to correlate with poor outcome of PC and SAP patients. Taken together, NGAL could be a novel marker with immense diagnostic and prognostic potential in AP and PC.

66 Plasma Proteome Analysis in Inflammation-Free Tgfb1–/– Rag2–/– Mouse Models of Human Colon Cancer

Durga Prasad Cherukuri[1], George Tsaprailis[1], Linda Breci[1], Constance Gard[1], Thomas Doetschman[1,2]

[1]BIO5 Institute; [2]Department of Cell Biology and Anatomy, University of Arizona, Tucson

Early detection of colorectal cancer (CRC) would significantly increase overall survival of individuals at high risk for CRC. Individuals having genetic predisposition to develop CRC, such as FAP and HNPCC patients, individuals having a family history of CRC (sporadic), or those with long-standing IBD, are at increased risk. HNPCC patients develop CRC at an age of approximately 45 years and undergo colonoscopy starting at age 25. Two-thirds of MSI-H HNPCC tumors are right-sided, but there is an association between colonoscopy and reduced mortality only in patients with left-sided cancer. IBD patients with extensive colitis undergo colonoscopy for detection of dysplasia starting at 8 years after IBD diagnosis, but they often develop CRC before the start of colonoscopy. Therefore, early cancer detection provides an opportunity for cancer chemoprevention in these high-risk patients. More than 90% of HNPCC CRCs and approximately 15–20% of sporadic and IBD CRCs exhibit MSI-H and marked genetic heterogeneity. Up to 70% of MSI-H sporadic and IBD CRCs and approximately 90% of HNPCCs exhibit TGFBR2 mutations. Therefore, identification of differentially expressed "markers"—early signs in blood that are associated with TGF beta deficiency—may help early detection of CRC in MSI-H patients with TGF beta-signaling deficiency. TGF beta-deficient mouse models provide a unique opportunity to identify differentially expressed plasma biomarkers associated with the absence of TGF beta signaling. Because TGF beta-deficient mice are dependent upon inflammation for the development of CRC, inflammation alone likely induces a variety of responses that could confound results from studies of TGF beta tumor suppressor function. Therefore, we performed plasma proteome analysis in inflammation-free Tgfb1–/– Rag2–/– mice and their littermate control Rag2–/– mice. Plasma pools from each group (n=3) were subjected to MudPIT analysis. We have identified 86 plasma proteins (each with at least two unique peptides) dysregulated between Tgfb1–/– Rag2–/– mice and their Rag2–/– controls at pre-tumor stages. Gene ontology analysis revealed that most proteins were extracellular, and approximately 30% of them are associated with transport, inflammation, and immune response. Further, we have identified a subset of 20 proteins relevant to colonic mucosa, and further validation will allow us to identify colon-specific plasma protein biomarkers that are indicative of TGF beta-signaling deficiency at both pre-tumor and tumor stages.

67 Cytokine Analysis of EUS-FNAs of the Pancreas Using Multiplex Immunoassays

William E. Grizzle[1], Nirag C. Jhala[1], Mohamad A. Eloubeidi[2], John D. Christein[3], Juan P. Arnoletti[3], Denise K. Oelschlager[1], Jin Wang[4], Qinghua He[5], Selwyn M. Vickers[6]

Departments of [1]Pathology, [2]Medicine, [3]Surgery, University of Alabama at Birmingham; [4]Auburn University, Auburn, AL; [5]Tuskegee University, Tuskegee, AL; [6]Department of Surgery, University of Minnesota, Minneapolis

Background: Endoscopic ultrasound fine needle aspirations (EUS-FNAs) are frequently used to diagnose masses of the pancreas. We have reported methods using immunohistochemical analysis to separate chronic inflammatory diseases, benign tumors, and neuroendocrine tumors from adenocarcinomas of the pancreas. This study was to determine whether the results of multiplex immunoassay of EUS-FNAs for cytokines would be useful as a diagnostic aid. **Method:** During the diagnosis of pancreatic masses using EUS-FNAs, patients were consented for obtaining an extra EUS-FNA for research. These were stored at -80°C neat or in 100 µl of 10% DMSO in RPMI 1640. EUS-FNAs were randomly selected from the collection to include 79 cases of pancreatic adenocarcinoma, 16 cases of neuroendocrine tumors of the pancreas, and 10 cases of benign pancreatic disease or normal pancreas. These EUS-FNAs were thawed in lysis buffer, diluted to samples with 500 µg/ml total protein, and analyzed in duplicate using a 27-plex kit that uses Luminex technology for multiplex immunoassays of the following cytokines: IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, FGF Basic, G-CSF, GM-CSF, IFN-g, IP-10, MCP-1, MIP-1a, MIP-1b, PDGFbb, RANTES, TNFα, and VEGF. **Conclusions:** In the separation of pancreatic adenocarcinomas from neuroendocrine tumors and/or non-neoplastic pancreatic diseases including cases with a normal examination of the pancreas, those cytokines which appear most useful are interleukin 1 receptor antagonist (IL-1ra), interleukin 6, and interleukin 8 ($p=0.012$ or less). In analysis of unprocessed plasma in a different set of patients using a multiplex assay that did not include IL-1ra, we previously identified that IL-6 and IL-8 were useful ($p<0.02$) in separating patients with adenocarcinoma from patients with non-neoplastic pancreatic diseases. These results suggest that the use of multiplex immunoassays of blood and of EUS-FNAs might be useful in the diagnosis of pancreatic cancer.

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68 Analysis of MicroRNA Expression in Sputum for Diagnosis of Lung Cancer

Feng Jiang[1], Nevins W. Todd[2], HongBin Fang[3], Min Zhan[3], Sanford A. Stass[1]

Departments of [1]Pathology, [2]Medicine, and [3]Epidemiology and Preventive Medicine, University of Maryland School of Medicine, Baltimore

Analysis of molecular genetic markers in biological fluids has been proposed as a useful tool for cancer diagnosis. MicroRNAs (miRNAs) are small regulatory RNAs that are frequently dysregulated in lung cancer and have shown promise as tissue-based markers for its prognostication. The aim of this study was to determine whether aberrant miRNA expression could be used as a marker in sputum specimen for the diagnosis of non-small cell lung cancer (NSCLC). Expressions of mature miRNA, mir-Let7a, was examined by real-time reverse transcription polymerase chain reaction (RT-PCR) and normalized to that of control miRNA, U6B, in sputum of 46 patients with NSCLC and 28 cancer-free subjects. The data were compared with conventional sputum cytology for the diagnosis of lung cancer. Mir-Let7a expression in the sputum specimens was significantly lower in cancer patients (42.59±8.13) than cancer-free individuals (59.35±6.72) (p=0.001). Furthermore, decreased mir-Let7a expression showed highly discriminative receiver-operator characteristic (ROC) curve profile, clearly distinguishing cancer patients from cancer-free subjects with areas under the ROC curve at 0.893±0.045. Detection of mir-Let7a expression produced 63.65% sensitivity and 100.00% specificity in diagnosis of lung cancer, as compared with 47.82% sensitivity and 100.00% specificity by sputum cytology. The measurement of altered miRNA expression in sputum could be a useful noninvasive approach for the diagnosis of lung cancer.

69 Serologic Biomarkers of B Cell Activation Prior to Lymphoma Diagnosis

L. I. Levin[1], E. C. Breen[2], B. M. Birmann[3], J. L. Kasperzyk[3], A. Marrogi[1], B. K. Kim[4], **O. Martinez-Maza**[2]

[1]Walter Reed Army Institute Research, Silver Spring, MD; [2]David Geffen School of Medicine at the University of California, Los Angeles; [3]Harvard School of Public Health, Boston, MA; [4]Armed Forces Institute of Pathology, Washington, DC

Serum levels of B cell-stimulatory cytokines and molecules associated with B cell activation are being examined in nested case-control studies of Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) that utilize stored sera from the Department of Defense Serum Repository (DoDSR). Our central working hypothesis is that elevated B cell activation, as evidenced by increased serum levels of B cell-stimulatory cytokines or molecules associated with B cell activation, precedes the appearance of these B cell cancers. In a study of HL, we assessed serum levels of IL6, IL10, sCD30, and IgE from more than 100 HL cases and matched controls (2:1 case control ratio) with multiple serum samples from the DoDSR collected up to the time of diagnosis. The median interval between the earliest serum collection and diagnosis date was 2.8 years (range: 1 day to 9.8 years). Median sCD30 and IL6 levels were significantly higher among cases than controls up to 1 year preceding HL diagnosis. These differences diminished with time, and no significant case-control differences in median levels were observed in the windows more than 1 year from diagnosis. An ongoing nested case-control study (funded by R01-CA121195), with serum samples retrieved from the DoDSR, aims to define longitudinally serum levels of these markers and other B cell activation-associated molecules preceding NHL diagnosis. In this study, we have identified approximately 700 B cell NHL cases, after querying the ACTUR (the military tumor registry) and the Armed Forces Institute of Pathology National Pathology Repository. This case list was submitted to the Armed Forces Health Surveillance Center (AFHSC), to determine case eligibility based on availability of retrievable pre-diagnosis serum samples. We are currently working with the AFHSC to finalize the case group, select matched controls, and obtain serial serum specimens for laboratory testing. We intend to assess serum levels of B cell stimulatory molecules using a combination of Luminex-based multiplexed assays and ELISAs.

70 Combined Biomarkers for Early Detection and Diagnosis of Ovarian Cancer

Anna Lokshin[1], Steven Skates[2], Aleksey Lomakin[3], Brian Nolen[1], Elieser Gorelik[1], Robert C. Bast, Jr.[4]

[1]University of Pittsburgh Cancer Institute; [2]Harvard University; [3]Massachussets Institute of Technology; [4]The University of Texas M. D. Anderson Cancer Center

Early detection of ovarian cancer has great promise to improve clinical outcome. Ninety-six serum biomarkers were analyzed in sera from healthy women and patients with ovarian cancer, benign pelvic tumors, and breast, colorectal, and lung cancers, using multiplex xMAPTM bead-based immunoassays. A Metropolis algorithm with Monte Carlo simulation (MMC) was utilized for analysis of the data. Analysis of a training set consisting of sera from 139 ovarian cancer patients with stages I-IIB cancer, 149 patients with stages IIC–IV, and 1,102 healthy women allowed identification of a 4-biomarker panel with 87% sensitivity (SN) for early stage and 94% SN for late stage ovarian cancer at 98% specificity (SP). This model was applied to an independent blinded validation set consisting of sera from 44 patients with early-stage cancers, 124 patients with late-stage ovarian cancer, and 929 healthy women providing unbiased estimates of 85% SN for early-stage and 94% SN for late-stage cases at 98% SP. This panel was selective for ovarian cancer showing SN=33% for benign pelvic disease, SN=6% for breast cancer, SN=0% for colorectal cancer, and SN=36% for lung cancer. Additionally, a separate 4-biomarker panel for discrimination of ovarian cancer from benign pelvic disease with 88% SN at 90% SP was identified and validated in an independent blinded set. We have further explored urine as a possible source of biomarkers. We have observed that several proteins with demonstrated association with ovarian cancer in serum are also differentially expressed in urine. The 4-biomarker panel consisting of combined serum and urine proteins offered 98% SN at 100% SP for late-stage disease. These data warrant further validation in larger validation set.

71 Prospective Study of Risk-Reducing Salping-Oophorectomy and Longitudinal CA-125 Screening Among Women at Increased Genetic Risk of Ovarian Cancer

Zoe Miner[1], Mark H. Greene[2], Philip J. DiSaia[3], Marion Piedmont[1], and Michael J. Birrer[4] on behalf of the Gynecologic Oncology Group

[1]Gynecologic Oncology Group Statistical and Data Center, Roswell Park Cancer Institute, Buffalo, NY; [2]Clinical Genetic Branch, National Cancer Institute; [3]University of California Medical Center, Irvine; [4]Massachusetts General Hospital, Boston

There is a need to develop an effective screening strategy for women at increased risk of ovarian cancer to help in their decision as to whether to undergo a risk-reducing salpingo-oophorectomy (RRSO). GOG-0199 has assembled two cohorts of women both at increased risk of ovarian cancer based on family history and/or mutations in BRCA1/2. One cohort chose to undergo RRSO and be seen by their physicians every 6 months, while the second cohort chose to be monitored by their physicians every 3 months without surgery. The study closed to accrual on November 2006 after enrollment of 2,605 patients. The primary objectives of the study include: (1) to determine ovarian cancer and other cancer incidence in this high-risk population; (2) to quantify the positive predictive value and specificity of the Risk of Ovarian Cancer Algorithm (ROCA); (3) to assess the quality of life between the two cohorts; and (4) to establish a longitudinal serum, plasma, and tissue repository for future evaluation. A broad array of both baseline and longitudinal data are collected and will be used to assess the interplay between patient behavioral characteristics and development of disease. Whole blood was collected at baseline and serum and plasma collected at each clinic visit from all patients. Fallopian tube and ovarian tissue was also collected from surgery patients. Whole blood was used to isolate DNA to test patients for BRCA1/2 mutations. Serum is being used for CA-125 testing, the results of which will be used in ROCA. Any remaining specimens will be banked for future research. More than 4,000 tissue specimens, 15,000 longitudinal serum specimens, 15,000 longitudinal plasma specimens, and 2,300 whole blood specimens have been banked so far from this valuable patient population. Survival analysis will be used to evaluate the 5-year cancer incidence rates between cohorts with stratification for mutation status. The screening algorithm will be evaluated by assessing two operating characteristics: positive predictive value and specificity, through hierarchical non-linear change-point models of longitudinal CA-125 values and use of Bayes' Theorem. Compliance to study measures has been good, particularly at baseline and within the first years. Ninety-seven% of patients received a baseline transvaginal ultrasound, 95% completed a baseline medical history questionnaire, 90% completed a baseline quality of life questionnaire, and 82% completed a 6-month health outcomes questionnaire.

72 DNA Methylation Markers for Colon Cancer Diagnosis

Yuriko Mori, Stephen J. Meltzer

The Johns Hopkins University School of Medicine, Baltimore, MD

In the United States, colorectal cancer (CRC) is the third most prevalent and deadly malignancy. CRCs are often diagnosed at advanced stages, due to the asymptomatic nature of early-stage CRCs. Screening of the average-risk population is crucial in order to reduce CRC death. Optimal population-based CRC screening requires a highly sensitive, specific, and noninvasive methodology. A candidate methodology is the detection of tumor-derived DNA in feces or serum utilizing cancer-specific DNA abnormalities (e.g., mutations or DNA hypermethylation events) as biomarkers. Several studies have tested biomarkers based upon known DNA abnormalities with well-established biological relevance to tumorigenesis. However, these biomarkers exhibited limited sensitivity because their occurrence tended to be specific to a certain carcinogenic pathway. Thus, optimal DNA biomarkers should be sought based upon their utility as biomarkers, rather than upon biological relevance. The objective of the current study was to develop optimal DNA hypermethylation-based biomarkers for population-based CRC screening. We conducted a genome-wide screening for putative loci that were hypermethylated in 17 primary CRCs relative to normal colonic mucosae obtained from 4 non-neoplastic control cases. The DNA microarray methodology, coupled with the methylated CpG island amplification methodology, was employed for this screening, which surveyed 55% of CpG islands within the genome. Fifty loci were prioritized for individual assessment based upon the following criteria: (1) array-based index of locus-specific DNA methylation level (methylation index) was greater in all CRCs than in any nonneoplastic controls; and (2) mean methylation index for CRCs was equivalent to that of the fully methylated standard DNA sample. Direct bisulfite sequencing-based methylation mapping of at these loci using pooled CRC and control DNAs was performed in order to identify regions with the greatest difference in methylation. By utilizing these mapping data, we designed quantitative real-time methylation specific PCR (Methylight) assays—a high-throughput, sensitive locus-specific methylation quantification methodology—for the 50 prioritized loci. Methylight evaluation is currently ongoing in a cohort comprising 43 sporadic CRCs, 50 colonic adenomas, and normal colonic mucosae from 73 non-neoplastic control cases.

73 Epigenome of Human Papillomavirus-16 in Head and Neck Cancers

Il-Seok Park[1,4], Myriam Loyo[1], Alice Chuang[1], Myoung S. Kim[1], William Westra[2], John R. Saunders[1,3], David Sidransky[1], **Sara I. Pai**[1]

Departments of [1]Otolaryngology/Head and Neck Surgery, [2]Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD; [3]Milton J. Dance Jr. Head and Neck Center at Greater Baltimore Medical Center, Baltimore, MD; [4]Department of Otolaryngology/Head and Neck Surgery, The Hallym University College of Medicine, Seoul, Korea

Human papillomavirus (HPV) DNA has been detected in the tumor nuclei of 60–70% of tumors localized to the oropharynx. HPV type 16 (HPV-16) is the viral type present in greater than 90% of HPV-associated head and neck cancers. HPV oncoproteins, particularly the E6 and E7 proteins, play critical roles in the viral transformation of the host cell. Once viral DNA is integrated into the host genome, it is possible for the virus to utilize the methylation machinery to induce viral gene silencing and facilitate viral escape from immune recognition. In order to determine whether the virus utilizes methylation as a mechanism of tumor immune evasion, we evaluated the DNA methylation status of the HPV genome in head and neck cancers. We designed contiguous primers to bisulfite treated sequences of the HPV-16 genome. Our primers were first tested on two human HPV-16 associated cancer cell lines, CaSki and Siha. We amplified 107 of the 110 individual CGs of the viral epigenome including the enhancer, the polycistronic early promoter, and the late promoter regions. The CaSki cell line was found to have significant methylation of the HPV-16 epigenome with only 4 unmethylated CGs. In contrast, the SiHa cell line demonstrated unmethylation of the long control region (LCR), the promoter region, and the enhancer regions. Subsequently, we sequenced the HPV-16 epigenome in advanced stage III/IV head and neck cancers. Interestingly, LCR, which regulates expression of the transforming proteins E6 and E7, was found to be unmethylated in all of the tumors. The enhancer region and the late promoter region demonstrated variable methylation status within the primary tumors. Methylation-specific polymerase chain reaction (MSP) was then performed to analyze the methylation status of the LCR, L1, and L2 regions in paired serum and saliva of head and neck cancer patients. The MSP results in serum and saliva correlated with the methylation status of the HPV-16 epigenome in the primary tumors. To our knowledge, this is the first effort to comprehensively sequence the epigenome of HPV-16 in human cancers. Thus far, we have been able to detect site-specific methylation patterns that relate to oncogenic viral gene expression. The methylation status of particular regions can provide further insight into the regulatory mechanisms of the virus, as well as potentially be used as diagnostic and prognostic markers in the serum and saliva of patients to monitor disease progression.

74 Immunohistochemical Validation of Genes Differentially Expressed Between Benign and Malignant Thyroid Tumors

Nijaguna B. Prasad[1], Jeanne Kowalski[2], Hua-Ling Tsai[2], Guennadi Kouniavsky[1], Kristin Talbot[1], Helina Somervell[1], William H. Westra[3], Douglas P. Clark[3], Christopher B. Umbricht[1], Alan P.B. Dackiw[1], **Martha A. Zeiger**[1]

[1]Department of Surgery, [2]Division of Oncology Biostatistics, [3]Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD

Background: It is well known that cytopathologists often experience difficulty preoperatively diagnosing thyroid lesions as either benign or malignant. As a result, 20–25% of fine needle aspirates (FNA) are reported as indeterminate or suspicious, and 10–15% are reported as inadequate for diagnosis. Because the clinician and surgeon cannot determine malignancy pre- or intra-operatively, patients with indeterminate thyroid lesions on FNA cannot be optimally clinically managed. Therefore, other adjuncts such as "molecular markers" are needed to differentiate benign and malignant thyroid nodules. With microarray analysis, we previously identified 75 molecular markers that are differentially expressed between benign and malignant thyroid tumors and validated 12 of the genes by real-time RT-PCR. In the present study, we have further validated the protein expression levels of 10 of the 75 genes in 154 thyroid tumors and 174 intra-operative FNA biopsy samples by immunohistochemistry (IHC) and immunocytochemistry (ICC), respectively. Methods and Results: We examined HMGA2, P-Cadherin/CDH3, DPP4/CD26, Stratifin/14-3-3σ, PLAG1, KLK7, CYP1B1, DIRAS3/ARH1, MRC2/Endo 180, and KIT protein expression in benign and malignant thyroid tumors. These 10 candidate genes were chosen from the list of 75 differentially expressed genes based upon their known association with thyroid or other cancers and the availability of commercial antibodies. We have analyzed each gene separately in all 154 tumors and further examined the association between tumor classes (benign versus malignant) and protein expression level (low-to-negative versus moderate-to-high) using a Chi-squared test. Nine of the 10 candidate-genes (HMGA2, P-Cadherin/CDH3, DPP4/CD26, Stratifin/14-3-3σ, PLAG1, KLK7, DIRAS3/ARH1, MRC2/Endo 180, and KIT) exhibited the expression levels that were significantly associated ($p<0.05$) with tumor class (benign versus malignant tumors) in both tissue-array and non-arrayed samples. We also examined the protein expression of one of the 10 candidate genes, HMGA2, in 174 FNA samples by ICC. Sixty-three (36%) had associated suspicious FNA cytology. Of the 174 samples (108 benign, 66 malignant), a statistically significant association was observed between HMGA2 expression level and tumor class ($p<0.0001$), with high/moderate expression associated with an increased odds of malignant (versus benign) tumors as compared to low/negative expression. Future Plans: We plan to examine protein expression of remaining 9-genes in a new set of thyroid FNA samples by ICC in anticipation of developing a diagnostic panel useful in the differential diagnosis of indeterminate or suspicious thyroid nodules.

75 Characterizing Inflammatory Cell Infiltrate as Biomarker for Cervical Cancer Screening: New Collection Media Permits Flow Cytometry and HPV Testing

Troy D. Querec[1], Martin Steinau[1], Daisy R. Lee[1], Mack T. Ruffin[2], **Elizabeth R. Unger**[1]

[1]Centers for Disease Control and Prevention, Atlanta, GA; [2]University of Michigan, Ann Arbor

Human Papillomaviruses (HPV) play an etiologic role in cervical cancer and have been used as screening biomarkers. Recently available HPV vaccines will not eliminate the need for screening as not all types of HPVs associated with cancer are targeted by vaccines, and the impact of vaccines on cancer incidence will not occur for 15–20 years after implementation. Screening for vaccine-missed cervical cancers will require even more efficient and cost-effective screening tools. Current screening methods, including for HPV, are inefficient. Less than one third of women referred for followup have preinvasive lesions requiring intervention (CIN 3). The host immune response is important in determining the outcome of HPV infection, and characterization of the inflammatory cell could potentially distinguish between women with simple infection and those with precancer lesions. We sought to apply flow cytometry to exfoliated cervical samples because the method permits a high enumeration rate of rare cells and simultaneous multiparameter quantification of cellular markers. We evaluated results on cervical samples collected in a methanol-based cervical cytology collection media (PreservCyt™ [Hologic, Inc., Bedford, Mass.]) and a media for preservation of blood for flow cytometry (Streck Cell Preservative™ [Streck, Omaha, Neb.]). The Streck Cell Preservative allowed detection of neutrophils and lymphocyte subsets that were not seen with the methanol-based samples. The Streck samples are stable at 4ºC for at least 1 week, allowing shipment of specimens from remote clinical sites. These Streck samples have been successfully used to detect and type HPV, indicating DNA is preserved. Because this new collection media is suitable for non-invasive cervical sampling, the host inflammatory response will now be able to be evaluated in epidemiologic studies. This method will have applications for sampling in other anatomic sites. We are also interested in testing the suitability of the preservative for other assay formats including RT-PCR, IHC, FISH, and proteomics.

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76 Plasma Glycoproteins as Biomarkers of Breast Cancer Patients

Fred E. Regnier, Wonryeon Cho, Kwanyoung Jung, Qiang Gao

Department of Chemistry, Purdue University, West Lafayette, IN

Aberrations of cellular glycosylation involving a small number of biosynthetic pathways are common phenotypes in cancer. These structural changes can alter the function of tumor cells, their antigenic and adhesive properties, and their potential to invade peripheral tissues and metastasize. Lewis x (Lex), sialyl-Lewis x (sLex), sulfosialyl-Lewis x (SsLex), and sialyl-Lewis a (sLea) antigens in various combinations are often elevated in the glycoproteins of cancer patients, along with increased β 1,6-branching, sialylation, and fucosylation of glycans. These changes are commonly observed during cancer progression in N- and O-linked glycoproteins on the surface of malignant cells. A critical element of metastasis is that malignant cells find a favorable site to bind and proliferate in remote organs. sLex on cell-surface glycoproteins gives malignant cells the ability to adhere to L-selectin on leukocytes, E-selectin and P-selectin on the vascular endothelium, and P-selectin on platelets. Lewis antigens can be bound at either N- or O-glycosylation sites on proteins, but core 2 O-glycans modified with sLex (C2-O-sLex) confer the highest binding affinity to selectins. Moreover, formation of C2-O-sLex glycans in breast cancer is enhanced by expression of the core 2 β(1,6) N-acetylglucosaminyltransferase (C2GnT) enzyme necessary for sLex synthesis. Although C2-O-sLex glycosylation plays a key role in metastasis, the proteins involved have generally not been identified. This is an important issue because proteins involved in metastasis could be biomarkers. As part of an ongoing effort to identify cancer biomarkers in plasma, we have identified a series of nine C2-O-sLex-carrying plasma glycoproteins that are elevated three fold or more in breast cancer patients. Analytical protocols are being developed and validated based on antibody selection of marker glycoproteins from plasma and confirmation of the presence of C2-O-sLex in appended glycans with a second antibody and mass spectrometry. The validated protocol will be used to screen small populations of breast cancer patients to verify the efficacy of these markers as diagnostic agents. Successful completion of this phase of the development process will prepare this cancer assessment modality for larger clinical validation studies.

77 Early Phase Development of Salivary Proangiogenic Cytokines as Biomarkers in Leukoplakia Clinical Trials

Nelson Rhodus[1], Eva Szabo[2], Beverly Wuertz[1], Kate Cole[1], Joe Rohrer[1], **Frank Ondrey**[1]

[1]Department of Otolaryngology and Masonic Cancer Center, University of Minnesota, Minneapolis; [2]Lung and Upper Aerodigestive Cancer Research Group, Division of Cancer Prevention, National Cancer Institute, Bethesda, MD

A significant emphasis has been placed on the potential utility of minimally or non-invasive surrogate endpoints in cancer clinical trials. Consequently, biomarkers analysis in saliva is a potentially attractive area of investigation in oral and other cancers. Our laboratory and others have demonstrated the upregulation of NF kappa B-dependent cytokines (IL-6, IL-8, and VEGF) in head and neck cancer cell lines, animal models, and oral cavity-derived fluids from patients. First, we identified IL-6, IL-8, and VEGF in relatively large concentrations in squamous cell line supernatants by ELISA. Next, we examined these cytokines in saliva and serum in oral leukoplakia patients. Twenty-one patients with preneoplastic leukoplakia were examined and lesions measured. We collected saliva, a 10 ml saline oral rinse, and serum on all patients before biopsy or treatment. Interleukin-6, -8, and VEGF were analyzed in triplicate by ELISA. We found that salivary cytokine levels were up to 10 fold higher than serum levels for all three cytokines. Interelukin-6 levels were much lower than the other 2 cytokines, similar to the cell line findings. IL-8 and VEGF were coordinately upregulated in saliva specimens ($p<0.0001$). Interestingly, there were moderate correlations (R squared .2–.4, $p<0.05$) between the size of the leukoplakia areas and the amount of cytokine produced once a single outlier was excluded. We conclude that NF kappa B-dependent cytokines that are produced by cancer and transformed oral cavity cell lines are also produced in the local milieu of oral leukoplakia. This study also suggests that the size of leukoplakia lesions appear to be related to the amount of cytokine produced in the milieu and serum. High levels of IL-8 and VEGF in leukoplakia patients are easily analyzed by standard ELISA technique, but IL-6 may need a more sensitive measure of analysis (e.g., bead technology). The correlative science between oral cavity cell lines and leukoplakia patients, with respect to cytokine secretion, strengthens the case for the further use of these salivary biomarkers in leukoplakia clinical trials (to monitor lesion size, response, etc.).

78 A Genomic Pathways Approach to Biomarker Discovery for the Early Detection and Risk Assessment of Pancreatic Cancer

Subrata Sen, Marsha Frazier, **Ann McNeill Killary**

Departments of Molecular Pathology, Epidemiology and Genetics, The University of Texas M. D. Anderson Cancer Center, Houston

In association with the National Cancer Institute Early Detection Research Network, we are taking a targeted genomic approach to identifying biomarkers for the early detection of pancreatic cancer. We hypothesize that genetic pathways initiated by loss of tumor suppressor genes within chromosome 3p and 1p and gain of oncogenes within chromosome 20q are critical determinants of pancreatic cancer and furthermore, by targeting these pathways, early biomarkers will be discovered. To identify a chromosome 3p pathway, 880 partial cDNAs were sequenced from a suppression subtractive hybridization library constructed to identify chromosome 3p12 pathway genes. Two additional expression platforms were screened including Affymetrix arrays interrogated with tumor/normal pancreatic samples to identify eight genes that are differentially expressed across the three expression platforms and verified by quantitative RT-PCR. Two of the eight genes, KSF-1 and -2, have been characterized to date and are secreted proteins. ELISA assays performed on 36 pancreatic cancer versus 19 normal control plasma indicated that plasma KSF-1 and KSF-2 levels were significantly higher in pancreatic cancer versus control plasma ($p<0.0005810$ and $p<0.000365$, respectively) with a specificity of 47% at 90% sensitivity and sensitivity of 25% at 90% specificity, AUC 0.7909 for KSF-1 and a specificity of 63% at 90% sensitivity and sensitivity of 64% at 90% specificity, AUC 0.8772 for KSF-2. In addition, we performed molecular profiling of both gene expression and genomic copy number on in vitro pancreatic cancer cell lines as well as tumor samples using multiple microarray platforms. An independent gene expression data set on normal pancreas, chronic pancreatitis, and adenocarcinoma was then used to analyze the profiles of the candidate genes and pathways identified. We have also identified, via the integrated genomic approach, a set of miRNAs potentially targeting the candidate genes, showing loss of function in the tumor samples. We have also profiled miRNAs in plasma and have demonstrated that expression profiles of a panel of four miRNAs discriminate pancreatic cancer plasma from healthy controls (sensitivity of 64% and specificity of 89%, AUC 0.82). Candidate biomarkers are also being examined for risk assessment in pancreatic cancer using single nucleotide polymorphism analysis. The overall goal is to develop a panel of biomarkers for the early detection and risk assessment of pancreatic cancer.

79 Evaluation of Complementary Biomarkers to CA125 at High Specificities for Ovarian Cancer Early Detection

Steven J. Skates[1], Eric Macklin[1], Ying Zhou[1], Allison Vitonis[2], Dan Cramer[2]

[1]Massachusetts General Hospital, Boston; [2]Brigham and Women's Hospital, Boston, MA

Due to the low annual incidence of disease, early detection of ovarian cancer requires very high screening specificity (99.6%) and high screening sensitivity (75% or greater) to achieve a minimum acceptable positive predictive value of 10%. Multiple CA125 screening studies have shown that a CA125 blood test followed by ultrasound for women with a positive blood test achieves the desired screening specificity if the blood test specificity is set at 98%. The focus for optimizing early detection of ovarian cancer then becomes the screening sensitivity when the blood test is set at 98% specificity. One approach to increasing screening sensitivity is identification of other blood biomarkers that complement CA125. Assessment of complementarity at high specificities (e.g., 98%) is not part of standard statistical methods such as logistic regression, which optimize results for the center of the distribution. We have developed a statistical mixture model to assess complementarity at high specificities that reflects the simple known biology of ovarian cancer biomarkers, namely that for any given biomarker, a proportion of tumors produce the marker and the rest do not. For ovarian cancers that do not produce a given marker we assume the distribution of the marker concentration in the blood is the same as the distribution in control subjects. Therefore, our statistical model for the distribution of the serum concentration of CA125 and a potential complementary biomarker (C) in patients with ovarian cancer is a bivariate Normal mixture with four components: (1) the cancer produces both CA125 and C; (2) the cancer produces CA125 and not C; (3) the cancer produces C and not CA125; and (4) the cancer produces neither biomarker. A similar four-component mixture of bivariate Normal distributions is used in the statistical model implemented for control subjects. Multiple biomarkers were measured in serum from ovarian cancer patients obtained prior to treatment and in control subjects matched for menopausal status, and an MCMC algorithm provided estimates of the parameters in the model. From these estimates the increase in sensitivity due to marker C at 98% specificity was estimated for HE4, CA72.4, and other biomarkers. The largest statistically significant increase was due to HE4. The point estimate for the increase was 5%. Discovery of another three markers with similar complementarity to CA125 as HE4 provides may be required before a substantial increase in sensitivity for ovarian cancer, such as 20%, is achieved. Ovarian cancer biomarker discovery should aim to identify biomarkers that complement CA125 at high specificities.

80 Serum Biomarkers for Breast Cancer Detection

Michael A. Tainsky[1,2], Ho-Sheng Lin[3,4], Jerzy Wojciechowski[1], Harvinder S. Talwar[4], Adi L. Tarca[5], Alexei Ionan[1], Madhumita Chatterjee[1], Bin Ye[1], Sorin Draghici[1, 5], Judith Abrams[6]

[1]Program in Molecular Biology and Genetics, Karmanos Cancer Institute, Detroit, MI; [2]Department of Pathology, Wayne State University, Detroit, MI; [3]Department of Surgery, Dingell VA Medical Center, Detroit, MI; [4]Department of Otolaryngology-Head and Neck Surgery, Wayne State University and Karmanos Cancer Institute, Detroit, MI; [5]Department of Computer Science, Wayne State University, Detroit, MI; [6]Integrated Biostatistics Core, Karmanos Cancer Institute, Detroit, MI

Background: The humoral immune response is a sensitive biosensor of overexpressed or novel proteins expressed by tumor cells and thus amplifies the signal of new protein changes in cancer. Panels of tumor antigens provide a sensitive and specific multianalyte immunoassay for the presymptomatic of cancer. Instead of single biomarker molecules, we use a high-throughput cloning method to identify panels of epitopes/antigens that react with autoantibodies to tumor proteins in the serum of patients with cancer. The binding properties of these serum antitumor antibodies on microarrays and advanced bioinformatics tools led to a panel of diagnostic antigens. **Results:** We have analyzed the serum IgG binding to the cloned antigens robotically spotted on nitrocellulose coated glass slides. Alexa 647 (Molecular Probes) red fluorescent dye was used to label an antibody to human IgG to detect patient serum IgG binding to clones on the microarray. We also normalized the red color intensity to Alexa 532 fluorescent intensity (green fluorescent dye) labeled anti-mouse IgG that reacts with the T7 capsid monoclonal antibody that binds to every T7 clone. The dye ratio data, red-over-green channel intensity ratios, were log-transformed, and the data were normalized to the print-tip group median within each array. The data were randomly split into training and testing set. For breast cancer we selected 91 antigen clones that were significant (false discovery rate adjusted $p<0.05$) with Mann-Whitney U-Test on the training set. Out of 91 clones, we selected the best combination of 6 clones with a Random Forest backward elimination algorithm that used out-of-bag error rate as a criterion. The model with 91 markers had an accuracy of 85%, and using 6 markers, the model achieved 80% accuracy on the independent testing set, which was not used in any previous part of the development of the classifier. For head and neck squamous cell carcinomas (HNSCC) the performance of this model for cancer classification on a training set consisting of 80 HNSCC and 78 control samples was assessed using ten-fold cross-validation repeated 100 times. Using this panel of 130 antigen markers on a completely new and independent set of 80 samples, an accuracy of 83.75% with sensitivity of 87.5% and specificity of 80.0% was achieved. Similar accuracy was achieved by reshuffling of the dataset and using other types of class-prediction models.

81 Induced Chromosomal Proximity and the Genesis of Gene Fusions in Prostate Cancer

Scott A. Tomlins[1], Javed Siddiqui[1], Shelia Aubin-Walker[5], Laurie Sefton-Miller[1], Radhika Lnu[1], Rohit Mehra[1], Nallasivam Palanisamy[1], David Wood[2], Lei Wang[1], Brent Hollenbeck[2], Yvonne Penabella[5], Stephanie Meyers[2], Harry Rittenhouse[5], Jack Groskopf [5], John T. Wei[2], **Arul M. Chinnaiyan**[1,2,3,4]

[1]Michigan Center for Translational Pathology; [2]University of Michigan Department of Urology; [3]Howard Hughes Medical Institute, University of Michigan Medical School, Ann Arbor; [4]Gen-Probe, Inc., San Diego, CA

Background: While a substantial number of men with serum PSA<4.0 ng/ml have prostate cancer, lowering PSA thresholds for biopsy would lead to an increase in negative biopsies and over-diagnosis of insignificant cancers. Recent evidence questions the utility of PSA screening for decreasing prostate cancer specific death. Here we evaluated TMPRSS2:ERG expression in post-digital-rectal-exam (DRE) urine for the diagnosis of prostate cancer.

Methods: TMPRSS2:ERG was measured by Transcription Mediated Amplification (TMA) in urine from 529 men referred for prostate biopsy at 3 institutions. Urine was collected from 79 men before prostatectomy. Results: In the University of Michigan needle biopsy cohort (n=201), TMPRSS2:ERG had superior AUC (0.75) compared to serum PSA (0.58) for prostate cancer diagnosis. Increasing TMPRSS2:ERG was associated with significant versus insignificant cancer on biopsy by Epstein criteria (n=78, p=0.04). Importantly, in men undergoing prostatectomy (n=79), increasing urine TMPRSS2:ERG was associated with upgrading on prostatectomy (6 to 7, p=0.05), high prostatectomy Gleason grade (6 versus >6, p=0.002) and large volume (<2 versus >=2 cm, p=0.03).

Conclusions: Measurement of TMPRSS2:ERG in post-DRE urine in men presenting for biopsy identifies clinically relevant prostate cancer at biopsy and prostatectomy. Additional studies in men with lower serum PSA are warranted.

82 EPS Markers in the Early Detection of Prostate Cancer

Christopher Whelan, Jarrod P. Clark, Kevin G. Chan, Mark H. Kawachi, David Josephson, Clayton Lau, Roger Satterthwaite, David D. Smith, Laura E. Crocitto, Ziding Feng, Timothy G. Wilson, Steven S. Smith

Division of Urology and Urologic Oncology, City of Hope, Duarte, CA

We developed a quantitative PCR test that detects both Type III and Type VI TMPRSS2:ERG fusions (Clark et. al [2008] Clinical Chem. 54:2007). The assay is quantified from a standard curve determined with a plasmid-cloned Type III TMPRSS2:ERG fusion target. In the study, expressed prostatic secretion (EPS) was collected under an IRB-approved, blinded, prospective study from 74 patients undergoing trans-rectal ultrasound-guided biopsy for prostate cancer. In addition to PCA3, TMPRSS2:ERG, and DNA methylation data, we collected data on PSA-RNA as part of the analysis of the data. We chose not to use it in the manner generally employed in PCA3 testing for three reasons. First, uncertainty in the marker-RNA/PSA-RNA ratios will be compounded because the errors associated with each parameter will sum to give the error in the ratio. Second, the excess mobilization of normal prostate cells that is expected from cancerous prostates was borne out by our data on RT-PSA values for PSA-RNA from EPS specimens. Third, G-run-associated secondary structures that form during the PCR reaction itself are not effectively suppressed by PCR additives. The characteristic performance of the test for Type III and Type VI TMPRSS2:ERG fusions in predicting biopsy outcome was compared with similar tests for the expression of PCA3 and DNA methylation levels at the APC, RARB, RASSF1A, and GSTPI genes. Each test improved characteristic performance over baseline DRE plus Serum PSA. However, the test for Type III and Type VI TMPRSS2:ERG fusions yielded the best performance in predicting biopsy outcome (AUC=0.823, 95% CI [0.728-0.919]) and Gleason's sum greater than 7 (AUC=0.844, 95% CI [0.740-0.948]). Available data do not permit characterization of TMPRSS2:ERG performance as a supplement to PSA and DRE testing; however researchers (Laxman et al. [2006] *Neoplasia* 8:885 and Laxman et al. [2008] *Cancer Res*. 68,645) have independently developed a TaqMan assay for Type III TMPRSS2:ERG fusions in post massage urine. Using a mixed cohort of diagnosed and undiagnosed patients, they report a sensitivity of 40.6% and a specificity of 72.9% for this single marker. This is to be compared with a sensitivity of 63% and a specificity of 80% using the assay developed by Clark et al. in EPS. These results suggest that the combination of plasmid standardized testing for TMPRSS2:ERG Types III and VI in EPS may be superior to testing for TMPRSS2:ERG Type III in post-massage urine. We will answer this question by making a direct comparison of the two approaches.

83 Role of SMRP and Osteopontin for Early Detection of Human Malignant Mesothelioma

Haining Yang[1], Harvey I. Pass[2], Michele Carbone[1]

[1]Cancer Research Center and Medical School Department of Pathology, University of Hawaii; [2]New York University School of Medicine and Clinical Cancer Center

Malignant mesothelioma (MM) is a very aggressive cancer that causes approximately 2,500 deaths per year in the United States. MM is incurable unless detected in its earlier stages. Only patients in the early stage 1A of disease, when therapy can be effective/curative, have survival rates of greater than 5 years. Unfortunately, less than 5% of MM patients are diagnosed at this early stage. Therefore, it is critical to develop novel approaches to identify for early detection those individuals at higher risk for MM among the exposed cohorts. Soluble Mesothelin Related Protein (SMRP) and Osteopontin are two promising biomarkers that have been linked to mesothelioma. It has been found that MM patients have high levels of osteopontin and SMRP. We tested the hypothesis that these biomarkers could allow detection of mesothelioma in the early stages by testing high-risk volunteers from the Cappadocian MM villages in Turkey. In those three villages, 50% or more of deaths are caused by mesothelioma. The very high mesothelioma incidence may allow us to obtain biomarker data in a relatively short time at a fraction of the costs required to follow a sufficiently large cohort in the United States that would produce the same incidence. We tested the feasibility of these studies in sera collected from mesothelioma-villagers in March and June 2006. We detected two early mesotheliomas in this prospective study. Moreover we tested sera from some villagers that were collected and frozen in 1994. Since then, 8 of 72 donors have developed mesothelioma. Of these, two patients had high SMRP and osteopontin levels several years before the disease was diagnosed. We are now still performing more studies and validating the specificity and sensitivity of these biomarkers for early detection. Presently the possible value of these markers as early detection markers is unknown, although our preliminary results support this hypothesis. If the study confirms our preliminary data, the results will be of great relevance and possibly benefit many million people exposed to asbestos and to other carcinogenic mineral fibers in the United States and worldwide.

84 Development of a Bladder Cancer-Specific Ligand Using a Combinatorial Chemistry Approach

Hongyong Zhang, Lanu Aina, Kit S. Lam, Ralph de Vere White, **Primo N. Lara Jr.**, Christopher Evans, Liang Cheng, James A. Bassuk, Chong-xian Pan

University of California, Davis Cancer Center

Most transitional cell cancers of the urothelial tract, typically of the urinary bladder, are diagnosed at a non-invasive, organ-confined stage. Non-invasive bladder cancer is ideal for targeted therapy because it is easily accessible through intravesical instillation, is relatively isolated from the rest of the human body, and has few confounding cells. Our group performed high-throughput screening of one-bead, one-compound combinatorial peptide libraries (OBOC) and identified one bladder cancer-specific ligand, heretofore called PLZ4. We discovered that this ligand can selectively bind to bladder cancer cell lines and primary bladder cancer cells from patients but not to normal urothelial cells, a normal cell mixture from bladder specimen, fibroblasts, or blood cells. This ligand can also bind to primary tumors of canine bladder origin, indicating that preclinical studies in large animals with spontaneously occurring cancer are potentially feasible. This ligand can also bind to tumor cells treated with urine at pH 6.0, but it does not bind to cells collected from the urine of a patient actively treated with intravesical Bacillus Calmette Guerin (BCG) therapy. Intravenous injection of PLZ4 linked to near-infrared dye Cy5.5 showed fluorescent uptake in mouse xenografts developed from excised primary tumors of bladder cancer patients. Thus this ligand also has the potential to be used for imaging detection of bladder cancer. Using alanine walk and our patented rainbow bead coding system, we determined the amino acids critical for cell binding. Structural analysis also indicated that there are two domains required for cell binding. PLZ4 has the potential to be used for imaging detection for diagnosis and followup/surveillance and perhaps as a delivery device for targeted therapy of urothelial (bladder) cancer.

85 Video Rate Optical Coherence Tomography for Early Stage Cancer Visualization

Paul Amazeen

Imalux Corporation, Cleveland, OH

Optical coherence tomography (OCT) is capable of visualizing microstructural tissue changes, in vivo, associated with cancer and other pathological conditions. We have developed an innovative combination of frequency domain optical coherence tomography (FD-OCT) and common path topology with a flexible endoscopic fiberoptic probe to produce a fast, practical, cost-effective system to survey large organs in real-time to aid in cancer detection at an early stage. For example, bladder cancer patients have a high 10-year survival rate but with a high rate of recurrence and progression; thus, there is a need for the development of more sophisticated surveillance tools. Currently, up to six random biopsies of normal appearing urothelium are taken in patients presenting with superficial bladder cancer at the time of the resection or biopsy of the visible tumor. Early clinical studies have demonstrated the potential to guide or replace biopsies, as well as guide surgical treatment. However, current OCT systems (<1 fps) have a slow image acquisition rate and can only perform single-point imaging; therefore, the ability to survey large surface organs like the bladder is limited and time-consuming. OCT can detect changes associated with bladder carcinoma or even precancer conditions like dysplasia. The ability to acquire OCT images at substantially higher rate (video rate) without compromising image quality would allow physicians/clinicians to survey large organs in an efficient amount of time. By detecting and treating additional foci of disease at a single point-of-care visit, video rate OCT during treatment could significantly decrease the recurrence rate of disease and thereby decrease the frequency of follow-up cystoscopy evaluations. This is a platform technology that can be applied to a number of medical specialties, and we have clinical evidence for applications in the diagnosis and treatment of diseases in gynecology (cervical cancer), otolaryngology (ENT: laryngeal cancer, oral cancer), and urology (bladder cancer). However, this is by no means an exhaustive list, and there is growing evidence of similar results in other medical applications. Clinical validation will impact the use of, and time delays associated with, a number of biopsies. Other modalities such as fluorescence can benefit from the increased specificity that OCT can add.

86 Non-Destructive Imaging to Monitor Colon Carcinogenesis

Jennifer Barton, Eugene Gerner, Marty Pagel, Russell Witte

The University of Arizona

As mouse models become more costly and complex, the need increases for non-destructive, time-serial evaluation of disease progression. Biomedical imaging has advanced to the point that rapid, minimally invasive visualization of internal structure and function of mouse tissues is possible with high resolution. We are developing novel imaging methods for non-destructive monitoring of colon cancer progression and response to treatment, augmenting information obtained at sacrifice. For example, imaging can enable differentiation of a tumor that progressed and then regressed under treatment to be distinguished from one that never progressed. Optical, ultrasound, and magnetic resonance imaging have been employed to cover the full range of imaging solutions needed, from ultrahigh resolution to full-body imaging. Endoscopic optical coherence tomography (OCT) has been combined with fluorescence imaging for ultrahigh (4–10 μm) resolution imaging of distal colon. Our studies have shown that tumor progression can be monitored over time in the AOM-treated mouse model and that OCT has very high sensitivity for small adenoma (95 percent) and can also identify gastrointestinal neoplasia. Optical methods have limited depth of imaging and require endoscopic implementation, whereas high frequency ultrasound has been used to visualize distal and proximal colon through the abdominal wall with excellent resolution (20–40 μm). Additionally, high-field, respiratory-gated MRI has been used to obtain whole-body imaging with 100 μm resolution. In all imaging systems, proper protocols for clearing and defining the colon lumen are critical for high contrast imaging. Image quality and information content can be increased by the use of contrast agents and imaging techniques that target specific tumor markers or provide information about vascularity and permeability.

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87 Genetic PET Imaging of HER2 mRNA to Detect Breast Cancer

Chang-Po Chen[1], Kaijun Zhang[2], Mathew L. Thakur[2,3], Brian D. Gray[4], **Koon Y. Pak**[4], and Eric Wickstrom[1,3]

Departments of [1]Biochemistry and Molecular Biology, and [2]Radiology, [3]Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA; [4]Molecular Targeting Technologies, West Chester, PA

Breast cancer attacked more than 275,000 women in the United States in 2007 and took the lives of more than 41,000 women. Mammograms detect abnormal lumps in breasts; over two-thirds of abnormalities are benign. The remaining one-third are malignant lumps consisting of rapidly growing breast cancer cells. The cells divide frequently due to mutational activation of cancer genes. If a lump is found, the next step is a biopsy to determine whether the lump is benign or malignant. Even if malignancy is confirmed, biopsies do not determine which oncogenes are activated and do not reliably diagnose estrogen-dependent versus estrogen-independent breast cancer. High levels of Her2 protein are associated with aggressive, estrogen-independent breast cancers. Measurements of mRNA demonstrated that Her2+ breast cancer cells express thousands of HER2 mRNAs per cell, while Her2- breast cancer cells express much less. We have designed and demonstrated a novel technology called genetic imaging to visualize active cancer genes from outside the body. In the clinic, we will scan the entire breast so that all sites of cancer gene activation can be seen, whether or not a lump has formed. Genetic imaging probes are peptide nucleic acid (PNA) sequences that hybridize specifically to messenger RNAs (mRNAs) copied from activated cancer genes. We added a small peptide analog to allow the genetic imaging probes to be taken up by the breast cancer cells. Finally, we chelated radionuclides to permit external imaging by positron emission tomography (PET) scanning. Genetic imaging probes for CCND1, IRS1, MYCC, and KRAS2 mRNAs, injected into animal models, enabled us to visualize breast cancer, pancreas cancer, and prostate cancer xenografts. We have designed, synthesized, purified, and characterized chelator-Her2 PNA-peptides. Radiolabeled Cu-64-chelator-Her2 PNA-IGF1 analogs showed three-fold greater binding to Her2+ BT474 cells than mismatch controls. Animal trials are underway in phase II.

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88 Development of an In Vivo Breast Cancer Detection Method Using SQUID-Relaxometry and Targeted Magnetic Nanoparticles

Edward R. Flynn[1], Natalie L. Adolphi[1,2], Robert Belfon[2], Howard C. Bryant[1], Kim Butler[2], Danielle Fegan[1], Helen J. Hathaway[2], Dale L. Huber[3], Jason E. Jaetao[1,2], Debbie M. Lovato[2], T. E. Tessier[1], Richard S. Larson[2]

[1]Senior Scientific LLC, Albuquerque, NM; [2]Departments of Pathology, Biochemistry, Cell Biology and Physiology, and Cancer Research and Treatment Center, University of New Mexico Hospital, Albuquerque; [3]Center for Integrative Nanotechnology, Sandia National Laboratory, Albuquerque, NM

A major goal in emerging technology for enhancing detection of cancer is the development of methods to identify tumors in the earliest stages of disease. For breast cancer, the current method of choice for screening and detection is mammography. While mammography has led to a significant improvement in our ability to detect breast cancer earlier, it still suffers from the inability to distinguish between benign and malignant lesions, difficulty in detecting tumors in dense and scarred breast tissue, and failure to detect 10–30% of breast cancers. The use of magnetic nanoparticles conjugated to tumor-specific reagents combined with detection of these particles through measurement of their relaxing fields following a magnetization pulse represents a promising new technology that has the potential to improve our ability to detect tumors earlier. Furthermore, detection of targeted magnetic nanoparticles using SQUID relaxometry is fast and theoretically more sensitive than MRI detection, because only particles bound to their target cells are detected, while localization and cancer cell density of the tumors is determined. Our group is developing conjugated magnetic nanoparticles targeted to breast cancer cells that express the Her2 antigen, which is overexpressed on approximately 30% of human breast cancers. Her2 antigen binding sites were quantified on several human breast cancer cell lines using a fluorescent-conjugated Her2 monoclonal antibody in a flow cytometry assay. Her2 antibody was conjugated to superparamagnetic iron oxide nanoparticles, and labeled nanoparticles were incubated with breast cancer cell lines with high (MCF7/Her2-18) or low (MDA-MB-231) levels of Her2 expression. Labeled cells were analyzed by microscopy and SQUID relaxometry, indicating an antigen concentration-dependent increase in the number of nanoparticles bound to cells. Breast cancer cells were inoculated subcutaneously into nude mice, and Her2 antibody-conjugated nanoparticles injected into the mice either intra-tumoral or by tail vein. The mice were then imaged in vivo by SQUID relaxometry. The measurements indicate that the Her2 antibody-labeled nanoparticles are retained in the tumor. Prussian blue labeling confirmed that iron-oxide-containing nanoparticles were detected in tumor tissue by histology. These results suggest that antibody-labeled magnetic nanoparticles have the potential to label breast tumor cells in vivo and, thus, present a new tool in breast cancer detection.

89 Microfluidic Live Cell Imaging System

Philip Lee, Terry Gaige, Paul Hung
CellASIC Corporation, San Leandro, CA

We have developed a microfluidics-based cell culture system for improved live cell imaging. This system complements advanced optical microscopy methods currently used for investigating the biology of living cancer cells (cell lines and primary cells). Key applications of this technology are in basic research, with emphasis on cell signaling pathways and time-varying responses. Current methods for live cell imaging rely on modified cover slides or bulky flow chambers. CellASIC has pioneered the use of advanced microfluidics technology to create continuous perfusion environments that promote cell culture and facilitate high magnification live cell microscopy. Key features include the ability to schedule exposures of different solutions during imaging, integrated temperature and CO2 control, reduction of cell/reagent usage, and compatibility with all inverted microscope setups. This allows any researcher to perform long-term (3+ days), time-lapse video microscopy from cell samples as small as 2 ul (approximately 1,000 cells). Additional benefits include multiplexing experiments in parallel, creation of stable chemical gradients, and defining complex flow exposure profiles.

90 Differences Between Ground Glass Opacities (GGOs) and Solid Nodules in CT-Scan Screening

Robert Lee, Dawn Walter, Yvonne Owusu-Sarpong, Ellen Eylers, David Naidich, **William Rom**
Division of Pulmonary and Critical Care Medicine, New York University School of Medicine, New York

Background: The New York University Lung Cancer Biomarker Center evaluates high-risk smokers who smoke over 20 packs per year with CT-scans. We hypothesized that lung cancers presenting as ground glass opacities (GGOs) or solid nodules (SNs) represent different points in time in the spectrum of cancer progression, or are separate entities with different prognosis, clinical course, and outcome.

Methods: Prospective data were obtained in a high-risk group of patients enrolled in the Early Detection Research Network (EDRN) trial between 2001 and 2008 using initial appearance on the CT-scan.

Results: Twenty biopsy-proven lung cancer nodules were identified in 19 patients out of 1,143 study subjects enrolled. There were no differences in mean age at enrollment and diagnosis, smoking history (54 pack-year versus 65 pack-year, p=0.24), asbestos exposure, initial size and absolute growth of the nodule, and stage of lung cancer. Differences were noted in the mean time to diagnosis (40 versus 17 months, p=0.03) and the mean number of CTs prior to diagnosis (7 versus 2, p=0.006) between the GGO and SN group, respectively. The FEV1 was lower in SN 1.95 (78%) versus GGO 2.46 (89%) and 5 out of 11 GGO had BAC, versus 0 of 12 SN.

Conclusion: Lung cancers presenting as GGO are more likely to have a delay in diagnosis with increased number of CT scans compared to those presenting with SN due to BAC histology.

91 Multicenter Selective Lymphadenectomy Trials

Donald L. Morton

John Wayne Cancer Institute at Saint John's Health Center, Santa Monica, CA

We introduced sentinel node biopsy (SNB) in the early 1990s to resolve controversy over elective lymph node dissection (LND) versus watchful waiting for patients (pts) with primary cutaneous melanoma. Early removal of regional node metastases improves survival, but only 20% of pts with clinically normal regional nodes have histopathologic evidence of nodal involvement. SNB is a minimally invasive technique to identify these pts without subjecting all pts to potential LND morbidity. SNB identifies and removes first tumor-draining lymph nodes (sentinel nodes [SNs]) on the afferent lymphatic pathway. Because focused SN histopathologic analysis accurately predicts tumor status of all nodes in a drainage basin, LND is undertaken only in pts with SN metastasis. Studies validate the SN concept not only for melanoma but also for many other solid tumors draining via lymphatics. SNB is a multidisciplinary technique: nuclear medicine physicians use preop lymphoscintigraphy; surgeons use intraoperative lymphatic mapping; and pathologists use IHC and molecular techniques. To determine feasibility of multidisciplinary SNB for widespread use, in 1994 we began the international Multicenter Selective Lymphadenectomy Trial (MSLT: A Clinical Study of Wide Excision [WEX] Alone Versus WEX With Intraoperative Lymphatic Mapping and Selective LND in the Treatment of Pts With Cutaneous Invasive Melanoma). Accrual completed in March 2002; 17 centers randomized 2,001 pts. MSLT data confirmed accuracy and minimal morbidity of SNB. SNB-based staging of intermediate-thickness (1.2 to 3.5 mm) primary melanomas provides important prognostic information and identifies pts with nodal metastases whose survival can be prolonged by immediate LND. Most (70%–80%) melanoma patients with SN micrometastases have no other tumor-involved nodes. Is LND still necessary? The second MSLT will answer this (MSLT-II: A Phase III Multicenter Randomized Trial of Sentinel Lymphadenectomy and Complete LND Versus Sentinel Lymphadenectomy Alone in Cutaneous Melanoma Patients With Molecular or Histopathological Evidence of Metastases in the SN). Currently, we have screened 2,102 pts (planned 4,200) at 43 sites in the United States, Canada, Israel, Europe, and Australia. We will randomize 1,925 tumor-positive SN patients to LND or to nodal observation with serial ultrasound of SN basin. MSLT-II began in 2004; 451 of 588 randomized pts have pathologic (H&E or IHC) evidence of SN metastasis, and 137 have molecular (multimarker RT-PCR, including MART-1 and MAGE-3) evidence of SN metastasis.

92 Proteomic Approaches for Development of Chemoprevention Targets

Masako Nakanishi[1], Antoine Menoret[2], Anthony Vella[2], Shingo Miyamoto[1], **Daniel W. Rosenberg**[1]

[1]Center for Molecular Medicine and Neag Cancer Center, [2]Department of Immunology, University of Connecticut Health Center, Farmington, CT

A complex interplay between genetic background and environmental factors is likely to influence the efficacy of intervention strategies for colon cancer prevention. One approach to optimizing cancer chemoprevention is to identify individuals who will be responsive to a particular agent. This may, in principle, be accomplished using genetic approaches, provided that genes important for a given biological response may be identified. On the other hand, a proteomic approach offers a number of distinct advantages, including the ability to detect post-translational modifications (PTMs) of proteins that play a fundamental role in generating a positive response (e.g., tumor suppression). In the following study, we describe the development of a proteomic-based approach to identifying tumor changes in real-time in response to chemoprevention treatment. It will be possible to determine which subpopulations of early colon lesions develop into tumors and whether chemoprevention agents suppress the rate of adenoma formation or promote their regression. Our method is predicted to recapitulate potential clinical scenarios, in which protein markers can be used to identify individuals with high-risk adenomas. In addition, our long-term goal is to customize chemoprevention in human populations based on expression of predictive proteins or genes uncovered in precancerous lesions. Our initial approach has been to characterize the response of ApcΔ14/+ mice to sulindac, a commonly used chemoprevention agent that has varying efficacy. We have adapted this strategy to resveratrol and black raspberry powder. In addition, we anticipate that our refinements in lesion imaging and feature recognition within the topography of the colon, established from mouse chromoendoscopy, may eventually be translated into the clinic as a procedure for monitoring the precise location of lesions that can then be followed longitudinally over time.

93 Photoacoustic Imaging—A New Modality for Sentinel Lymph Node Mapping in the Staging of Breast Cancer

Michael Pashley[1], Lihong Wang[2], Ladislav Jankovic[1], Todd Erpelding[1], Kim Chul-Hong[2], Konstantin Maslov[2]
[1]Philips Research, Briarcliff Manor, NY; [2]Washington University, St. Louis, MO

Sentinel lymph node biopsy (SLNB) has become the standard method of axillary staging for patients with breast cancer and clinically negative axillae. Radioactive tracers (Tc-99 colloids) are used to identify the general area of the SNL, and methylene blue dye injected at the tumor site provides direct visualization of the SNL with an invasive surgical procedure. Once the SNL is identified, it is surgically removed for pathology. Even though this procedure has a high identification rate, it relies on a surgical procedure with associated morbidity. Ultrasound imaging is able to evaluate axilla morphology but cannot identify the SLN. We are developing a combined ultrasound and photoacoustic imaging modality that will be able to image morphology as well as directly identify the SNL. Photoacoustic imaging provides contrast based on optical absorption with the resolution of ultrasound imaging and is readily able to image methylene blue dye. We are therefore able to directly image the SNL following injection of methylene blue dye without the need for radioactive tracers or surgical intervention. This will allow noninvasive diagnostic methods such as fine needle aspiration biopsy to stage the axilla. Eventually, photoacoustic molecular imaging may be able to identify metastases in situ, removing the need for needle biopsy, and monitor therapy as well. We have developed a combined ultrasound and photoacoustic imaging system based on a commercial Philips iU22 ultrasound scanner. We are able to collect real-time images, overlaying both conventional ultrasound and photoacoustic contrast. We have successfully demonstrated the ability to image sentinel lymph nodes in a small animal. The photoacoustic signal from the dye is significantly stronger than from blood, allowing lymph vessels as well as nodes to be easily seen after the injection of methylene blue dye. Optimization of the system is in progress in preparation for initial clinical evaluation. In this paper we will present our latest results and include an overview of the supporting activities in the Washington University NTR.

94 Spectroscopic Rectal Microvascular Detection for Colorectal Neoplasia Risk Stratification

Hemant K. Roy[1], Andrew Gomes[2], Michael Goldberg[1], Sarah Ruderman[2], Jeremy Rogers[2], Valentina Stoyneva[2], Laura Bianchi[1], Eugene Yen[1], Mohammed Jameel[1], Andrej Bogojevic[1], Vadim Backman[2]
[1]NorthShore University HealthSystem, Evanston, IL; [2]Northwestern University, Evanston, IL

Background: Colonoscopy has the ability to reduce future colorectal cancer (CRC) risk by 75–90 percent. However, more than 90 percent of all screening colonoscopies are negative for clinically significant neoplasia (advanced adenomas or carcinomas), highlighting the need for risk stratification. One promising approach has been to exploit the "field effect," which is well-established phenomena in colon carcinogenesis. Our multidisciplinary group has developed novel optics technology, polarization-gated spectroscopy (4D-ELF) (Gastro, 2004) that allows accurate quantification of the colonic micro-circulation. We have previously demonstrated that in experimental models of colon carcinogenesis there is an early increase in blood supply (EIBS) that is confined to the peri-cryptal capillary plexus and precedes any microscopic abnormality (Gut 2005). We confirmed the occurrence in humans with a novel endoscopically compatible fiberoptic 4D-ELF probe (Gastro 2008). In the current study, we examine the ability of rectal EIBS to identify patients harboring neoplasia.

Methods: Two hundred and sixteen unselected patients undergoing screening/surveillance colonoscopy at Evanston Hospital had five readings (each taking approximately 50 milliseconds) taken from the endoscopically normal rectal mucosa, including microvascular blood content within 100 micron of tissue surface with specific attention to the oxygenated hemoglobing (Ohg) and packaging length scale (PLS), a measure of effective blood vessel volume.

Results: Using a single marker, rectal OHb was increased by approximately 55 percent in the patients with advanced adenomas (AA) elsewhere in the colon regardless of the location (proximal and distal AA were equivalent). This effect dissipated at the penetration depths of over 95 microns and was not evident with smaller adenomas. The AUROC for AAs was 0.88, and leave-one-out cross validation had AUROC=0.84. Similarly, PLS was altered only in patients who harbored AAs and had an additive diagnostic effect with the combination yielding an AUROC of 0.91 for AAs.

Conclusions: We demonstrate that measuring rectal pericryptal microvascular blood content for the endoscopically normal mucosa allows identification of patients at risk of harboring clinically significant neoplasia elsewhere in the colon. If validated by future trials studies, we would envision development of a free-standing probe that could be coupled with the annual rectal exam in order to enable the primary care physician to recommend the timing and intrusiveness of future CRC screening.

95 Blocking Tumor/Macrophages Cross-Talk With Anti-Mannose Receptor Recombinant Antibodies to Trigger Tumor Rejection

Nathalie Scholler[1], Denarda Dangaj[1,2], Raphael Sandaltzopoulos[2], and George Coukos[1]
[1]Ovarian Cancer Research Center, Department of Obstetrics and Gynecology, School of Medicine, University of Pennsylvania; [2]Department of Molecular Biology and Genetics, University of Thrace, Greece

Macrophages are an essential constituent of the tumor microenvironment, and their presence has been correlated with increased angiogenesis and tumor growth. Tumor microenvironment polarizes macrophages toward a tumor-associated phenotype (TAM) or M2 that overexpresses mannose receptor (MR) and downregulates secreted levels of IL-12. Independent works showed that MR cross-linking with anti-MR monoclonal antibody causes DC differentiation into APC promoting T-cell anergy and suggested that macrophage phenotype switch is linked to a "chemical conversation" between tumor and macrophages through exchange of soluble mediators. Yet, exact mechanisms underlying macrophage phenotype switch remain to be elucidated. To test whether soluble glycoproteins released by tumor cells could cause macrophage polarization toward M2 through binding to MR, we generated anti-MR recombinant antibodies (scFv) and assessed their function on macrophages in an in vitro cell model system. We established a model system of cell co-culture in transwell allowing chemical exchanges between human monocyte-derived M1 or M2 macrophages and human ovarian cancer cell line OvCar3 that expresses mesothelin, a soluble glycoprotein. We first confirmed macrophage phenotype switch from M1 to M2 after 3 days of co-culture with OvCar3 cells. We then showed by flow cytometry analysis that tumor-released mesothelin could bind to macrophage cell surface and that binding was greater on M2 than on M1 macrophages. Second, we generated a novel yeast-display recombinant antibodies (scFv) library from human origin that we screened with a yeast-expressed MR recombinant protein, and we identified three scFv against MR. We monitored phenotype switch of M1 macrophages toward M2 during co-culture with tumor cell in presence or in absence of mannan, a ligand for MR, or of anti-MR scFv. By flow cytometry and qRT-PCR we showed that all the novel anti-MR scFv could completely or partially prevent binding of tumor-released mesothelin to macrophages, and we demonstrated that one anti-MR scFv could prevent macrophage polarization toward M2 during co-culture with tumor cells. Our findings indicate for the first time that mesothelin glycoprotein binds to MR and that tumor-induced M2 macrophage polarization can be blocked with anti-MR scFv. Our results also suggest that tumor-secreted glycoproteins contribute to M2 macrophage polarization through MR binding, which could be another strategy developed by tumors for immunomodulation. Finally, we propose that anti-MR scFv could prevent macrophage phenotype switch in vivo, allowing to block or delay tumor growth.

96 Fat-Water Ratio and Diffusion-Weighted MRI Applied to the Measure of Breast Density as a Drug Response Biomarker for Chemoprevention Studies

Patricia A. Thompson[1,2], Veronique Poulin[1,2], Maria Altbach[1], Matthew Kupinski[1], Denise Roe[1,2], Theodore Trouard[1], Per Granstrom[1], Georgette Frey[1,2], Scott Squire[1], Alison T. Stopeck[1,2]
[1]University of Arizona, Tucson; [2]Arizona Cancer Center, Tucson

In spite of strong preclinical evidence for a number of promising prevention agents for the breast, advances in breast cancer chemoprevention are limited by lack of a quantifiable and non-invasive intermediate biomarker that informs on agent response and risk modulation. Results from the IBIS-1 trial of tamoxifen (TAM) provide the first evidence that the cancer prevention activity of TAM is mediated, at least in part, through effects on "mammographic density." Low precision with poor reproducibility, sensitivity, and accuracy, however, impede the use of mammographically-determined density as a drug response biomarker for screening novel agents.We used a novel radial gradient and spin-echo (radial-GRASE) MRI pulse sequence in combination with a fat-water decomposition algorithm to obtain fat-water ratios (FWR) with correction for effect of field inhomogeneities. The method is referred to as FWR-MRI. In addition, we collected diffusion-weighted MR images (DW-MRI). Acquisition time for the FWR-MRI and DW-MRI sequences was less than 5 minutes, and contrast was not utilized. The costs for all required MR scans approximated those for standard mammography. A total of 25 women were studied, including 8 with no history of breast cancer, 15 with breast cancer, and 2 at high risk for breast cancer. All subjects were ascribed a BI-RAD score and percent density based on mammogram. Histograms of subjects with less-dense breasts were single-peaked, and histograms from subjects with dense breasts were dual-peaked. We chose initially to look at the fraction of pixel values (Frc) below 30, 40, and 50 percent fat. For Frc 30, 82 percent of variance was between-subjects, 11 percent was between-sides within the same subject, and 7 percent was between-replicates within same side and subject. Intraclass correlation coefficient was highest for Frc 30. Highly significant relationships ($p < 0.001$) were observed for all three measures of FWR-MRI (Frc 30, Frc 40, Frc 50) and mean apparent diffusion coefficient (ADC) values, percent density by mammography ($p < 0.001$), and mammographic BI-RADS score ($p < 0.001$). Analysis of data obtained over entire breast was more predictive than assessment of only largest slice (in terms of volume). Menopausal status (post- versus pre-) was strongly related to FWR-MRI values ($p < 0.001$).These data illustrate utility of FWR-MRI and DW-MRI to accurately measure breast density. Studies are ongoing to determine if early changes in FWR or DW-MRI can be observed in response to chemoprevention agents.

97 NSAIDs in Cancer Detection

Jashim Uddin[1], Brenda Crews[1], Anna Blobaum[1], Philip Kingsley[1], David Piston[2], **Lawrence J. Marnett**[1]

[1]Departments of Biochemistry, Chemistry, Pharmacology, and [2]Molecular Physiology and Biophysics, Vanderbilt Institute of Chemical Biology and Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, TN

Non-steroidal anti-inflammatory drugs (NSAIDs) have demonstrated utility in cancer prevention and therapy in a range of settings. These drugs act by inhibiting the action of cyclooxygenase (COX) enzymes, which catalyze the first step in the biosynthesis of prostaglandins. COX-2 is a particularly important target because it is a major contributor to the inflammatory response and cancer progression and is inhibited by both non-selective NSAIDs and COX-2 inhibitors. COX-2 is absent or expressed at low levels in most epithelial cells but is found at high levels in inflammatory lesions and many premalignant and malignant tumors. COX-2 inhibitors have demonstrated significant efficacy in human clinical trials for prevention or adjuvant treatment of cancer, but their use is complicated by the occurrence of adverse cardiovascular effects in long-term, placebo-controlled trials. The high levels of COX-2 in premalignant and malignant tumors compared to surrounding normal tissue suggests it is an ideal target for molecular imaging. We synthesized a series of novel fluorescence imaging agents that efficiently target COX-2 in inflammatory lesions and tumors. A critical feature for the design of COX-2-targeted agents is our observation that amide derivatives of certain COX inhibitors are slow, tight-binding inhibitors of COX-2 but not COX-1. A library of compounds was prepared by the attachment of fluorophores through different tethers to indomethacin. Compounds were evaluated for COX-2 inhibitory activity against purified enzyme, macrophages, and tumor cells. Potent and selective inhibitors also were tested for their ability to image COX-2 in macrophages and tumor cells. The most effective compounds were conjugates with carboxy-X-rhodamine derivatives. Introduction of these reagents by intraperitoneal or intravenous injection provides sufficient signal for in vivo fluorescence imaging and high levels of accumulation in inflamed or tumor tissue compared to normal tissue. Experiments with COX-2 (-/-) animals or with animals pretreated with indomethacin or celecoxib verified that selective accumulation into inflamed tissue or tumors was due to binding to COX-2. Pharmacokinetic analysis revealed that only the intact parent compound is found in the region of interest. Because of their high specificity, contrast, and detectability, these COX-2 beacons are ideal candidates for detection of inflammatory lesions or early stage COX-2-expressing human cancers, such as those in the esophagus, oropharynx, and colon.

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98 Nanoscale Delivery Vehicles for Cancer Immunotherapy

Tristan T. Beaudette[1], Kyle E. Broaders[1], Joel A. Cohen[1], Edgar G. Engleman[2], Jean M. J. Fréchet[1]

[1]College of Chemistry, University of California, Berkeley; [2]Department of Pathology, Stanford University School of Medicine, Palo Alto, CA

Multiple factors contribute to the failure of dendritic cells (DCs) to prime effective anti-tumor T cell responses in tumor-bearing hosts. DCs can be manipulated in various ways to better stimulate anti-tumor immunity for use as therapeutic cancer vaccines. However, the majority of these approaches rely on the ex vivo manipulation of autologous DCs. We have investigated another approach for the loading of DCs with antigen without the need for ex vivo manipulation. We began designing and synthesizing particulate carriers capable of carrying a payload of antigen and also targeting and activating DCs in vivo. An ideal vehicle for antigen-based vaccines must be capable of delivering antigen into antigen presenting cells for efficient class I and class II antigen presentation while simultaneously activating elements of the innate immune system.To meet this challenge, we first developed a model delivery vehicle platform based on protein-loaded acid-degradable polyacrylamide hydrogel particles and have demonstrated that these particles significantly enhance MHC class I presentation and CD8+ T cell activation. We were able to functionalize these particles by incorporating cationic monomers and antibodies for enhanced uptake, or immunostimulatory CpG DNA to induce DC maturation. Additionally, these particles were very effective in tumor protection experiments carried out in mouse models. While these polyacrylamide particles were never intended for long-term use, they were key to the proof-of-concept demonstration that acid-degradable particles are promising for antigen-based vaccines. During our proof-of-concept studies, we simultaneously developed several polymer carriers more suitable for clinical use. After significant biological testing, we have selected a recently developed material prepared from the FDA-approved polysaccharide dextran for further development. Beyond its performance in vaccine formulations, this material is extremely promising for clinical translation due to its simplicity, scalability, and tunability. Under acidic conditions (i.e., in phagolysosomes), these new particles degrade to release encapsulated antigen and immunostimulatory agents, native dextran, and innocuous small molecule byproducts. Preliminary work suggests that these particles are highly tunable, at least as effective, if not more so, than our proof-of-concept system, and easily functionalized with targeting groups.

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99 Results of a Phase 1 Trial With Intravesical Ad-IFN-α/Syn3 for Superficial Bladder Cancer Including Putative Marker Studies

William F. Benedict[1], Mark B. Fisher[1], David L. Cutler[2], Abraham Alice[1], Sophia Young[2], Zhibo Yang[1], Michael A. O'Donnell[3], Colin P. Dinney[3]

[1]The University of Texas M. D. Anderson Cancer Center, Houston; [2]Schering-Plough Research Institute, Kenilworth, NJ; [3]The University of Iowa, Iowa City

Intravesical Bacillus Calmette-Guérin (BCG) is currently the gold standard for the treatment of recurrent superficial bladder cancer. Despite BCG and other second-line therapies, recurrence is a significant problem. Intravesical gene therapy may be a viable alternative treatment modality. The results obtained from the first 15 patients enrolled in a Phase I, non-randomized, multi-center, open-label intravesical gene therapy trial utilizing adenoviral mediated interferon alpha (Ad-IFNα) are presented. Patients were those with BCG refractory superficial bladder cancer (CIS/pTa). Patients received 75 ml in escalating doses of Ad-IFN (3x10E9, 1x10E10, 3x10E10, 1x10E11, and 3x10E11 particles/ml) with 1mg/ml of Syn3 used as an excipient to increase transfection. Pre-treatment, 0–24 hour, 24–48 hour, and first voided urine specimens on days 3–7, 10, and 14 were tested for IFNα levels. Levels of TRAIL as well as M65 and M30 (the latter of which are putative markers of necrotic and apoptotic cell death, respectively) were also examined. Intravesical administration of Ad-IFNα/Syn3 was found to be safe with only initial urinary urgency reported which was controlled with anti-cholinergic medication. Urinary IFN levels for patients receiving 3x109 Ad-IFNα were below the lower limit of the assay (156pg/mL). Two patients receiving 1x1,010 Ad-IFNα had measurable levels of urinary interferon with expression ranging from 2 to 6 days with peaks of 776 pg/mL and 1,038 pg/mL. Patients receiving 3x10E10 Ad-IFNα had detectable IFN for 3 to 6 days with peak levels ranging from 4690 to 10,568 pg/mL. Patients receiving 1x10E11 Ad-IFNα had detectable IFN for 4 to 6 days and peak levels ranging from 2,138 to 4,689 pg/mL. The first patient treated with 3x1,011 particles/ml (Pt. 14) had a peak level of 13544 pg/ml in the 0–24 hour urine collection with the 7 day level of 1,543 pg/ml. A peak level of 10,936 pg/ml was seen in Patient 15's 0–24 hour urine with a level of 1,769 seen at 4 days. After retreatment of Patient 14 at 90 days following the first treatment, high levels of IFN were also found in the 0–24 hr urine (6,092 pg/ml). Significant and prolonged levels of TRAIL, M65, and M30 were seen in many patients as well following Ad-IFN treatment. Of the 15 evaluable patients, 7 had complete remissions (CRs), defined as a negative cytology and biopsy at 3 months, and the CRs have been maintained to date up to 21 months.

100 Using Cancer Vaccines to Modulate Receptor Biology, Augment the Activity of Signaling Inhibitors, and Kill Tumors

Timothy M. Clay[1,2], Michael A. Morse[3], Gayathri R. Devi[1,4], Wei Chen[3], H. Kim Lyerly[1,2,4]

Duke Comprehensive Cancer Center and Departments of [1]Surgery, [2]Immunology, [3]Medicine, and [4]Pathology, Duke University Medical Center, Durham, NC

We are developing a series of recombinant viral vector-based cancer vaccines that are currently in late-stage preclinical studies or being tested in Phase I clinical trials. These vaccine platforms build upon our prior experience with other vaccination strategies, including dendritic cell-based immunotherapy, and appear to be potent stimulators of T and B cell responses, even in the presence of preexisting vector immunity. We have enhanced the vaccine efficacy using novel vector constructs and observed potent T cell and antibody-mediated immune responses in pre-clinical animal models. A major focus of these studies is the role of the tumor-specific antibodies induced by our vaccines (vaccine-induced antibodies), which exhibit both classical immune-mediated tumor cell killing by ADCC and CDC, but also inhibit tumor proliferation and augment the activity of small molecule signaling inhibitors. This novel insight into antibody-mediated effector mechanisms could improve our understanding of how immunotherapy impacts tumors and may lead to more effective cancer therapies by combining vaccines targeting growth factor receptors and small molecule signaling inhibitors.

101 Spontaneous Myelogenous Leukemia in Genetically Defined Miniature Swine: Potential for Development of a Clinically Relevant Large Animal Tumor Model to Assess Tumor Immunotherapy

Raimon Duran-Struuck, Patricia S. Cho, Alexander G.S. Teague, Shannon G. Moran, Isabel Hanekamp, David H. Sachs, **Christene A. Huang**

Transplantation Biology Research Center, Massachusetts General Hospital, Boston

The Massachusetts General Hospital (MGH) miniature swine characterized at the major histocompatibility complex (MHC) serve as a model system for human clinical transplantation. Sublines of these animals have been successfully inbred and now permit adoptive transfer studies in a large animal pre-clinical model. We report here five cases of spontaneous chronic myeloid leukemia with similarities to human disease that have occurred in our inbred miniature swine herd. Four of these cases occurred within the highly inbred histocompatible sublines. All animals presented with symptoms of weight loss, lethargy, and marked leukocytosis. Samples were obtained for complete blood count, peripheral blood smear, and flow cytometry analysis. Animals that were confirmed to have neoplasms were then euthanized. At necropsy, all animals had diffuse disease involvement of vascularized organs and severe hepatosplenomegaly. Peripheral blood mononuclear cells and tissues were phenotyped via fluorescence-activated cell sorting (FACS). In addition, all affected tissues were assessed histopathologically. Taking under consideration the clinical presentation, FACS, gross pathology, and histopathology we diagnosed these cases as chronic myelogenous leukemias. Some of these tumors have been successfully established as in vitro cell lines and are candidates to become the first leukemias to be utilized as transplantable tumors in inbred and MHC-defined large animals. Thus, MGH MHC-inbred miniature swine and established porcine tumors may provide useful preclinical translational models to study immunotherapeutic approaches to treat human leukemia.

102 In Vitro and In Vivo Recognition of Head and Neck Cancer Stem Cells by Aldehyde Dehydrogenase 1 Family Member A1-Specific CD8+ T Cells

Soldano Ferrone, Carmen Visus, YangYang Wang, Miroslaw Szczepanski ,Theresa L. Whiteside, Robert L. Ferris, HuiXui Wang, Albert B. DeLeo

University of Pittsburgh Cancer Institute, Pittsburgh PA

Cancer stem cells (CSC) are thought to be resistant to chemo- and radiotherapy. This emphasizes the need for the development of novel therapeutic strategies targeting CSC. Based on our initial evidence that CSC are more sensitive to immune effector T cells than tumor cells, we have focused on the development of immunotherapy for CSC. In human breast carcinomas or head and neck squamous cell carcinomas (HNSCC), CSC can be identified by flow cytometry as ALDH-bright cells based on elevated expression of aldehyde dehydrogenase 1 family member A1 (ALDH1A1). ALDH-bright cells sorted from the HNSCC PCI-13 cell line are tumorigenic in immunodeficient mice at a low inoculum of 500 cells. These ALDH-bright cells are recognized in ELISPOT IFNγ assays by the ALDH1A1-specific, HLA class I-restricted CD8+ T cells we have established. ALDH-neg cells sorted from PCI-13 cell line are not sensitive to ALDH1A1-specific effector cells unless pulsed with exogenous peptide. Bulk tumor cells were only sensitive to the CTL after pre-treatment with IFN-γ to upregulate HLA class I molecules and the antigen presenting machinery (APM) component expression in tumor cells. In contrast to the bulk population of tumor cells or ALDH-neg subpopulation of PCI-13 cells, ALDH-bright cells expressed higher levels of HLA class I and II antigens. They also had higher expression levels of (APM) components TAP1, TAP2, and tapasin. We surmise that the upregulation of ALDH1A1 and APM components results in an enhanced presentation of HLA-A2/ALDH1A188-96 peptide complexes in CSC, permitting efficient T-cell recognition. These in vitro results are paralleled by the ability of adoptively transferred ALDH1A1 peptide-specific CD8+ T cells to selectively eradicate ALDH-bright cells in PCI-13-derived xenografts progressively growing in immunodeficient mice. Our results demonstrating the selective sensitivity of ALDH-bright CSC to ALDH1A1 peptide-specific CD8+ T cells emphasize the potential of employing ALDH1A1-based immunotherapy to eliminate CSC and thus to better control tumor growth in patients with SCCHN and, by extension of this concept, in patients with other solid tumors.

103 A Phase 1 Trial of Repeat Intrapleural Adenoviral Interferon-Beta in Malignant Mesothelioma and Malignant Pleural Effusion Patients

Andrew R. Haas, Daniel H. Sterman, Edmund Moon, Adri Recio, and Steven M. Albelda

University of Pennsylvania Medical Center, Philadelphia

In a previous study, we demonstrated that a single intrapleural dose of an adenoviral vector expressing interferon-ß (Ad.IFN-ß) in malignant pleural mesothelioma (MPM) or malignant pleural effusion (MPE) patients was well tolerated and resulted in gene transfer, significant humoral immune responses, and some clinical responses. Based on preclinical data demonstrating enhanced efficacy with multiple Ad.IFN-ß doses, this Phase I trial was conducted to determine the safety, gene transfer efficiency, and immunologic and clinical responses of two intrapleural Ad.IFN-ß vector (BG00001) doses. Seventeen patients (10 with MPM, 7 with MPE) received 2 Ad.IFN-ß doses administered through an indwelling pleural catheter in doses ranging from 3 x 1,011 to 3 x 1,012 viral particles (vp). Subjects were evaluated for (1) toxicity, (2) generation of adenoviral neutralizing antibodies (Nab), (3) gene transfer, (4) humoral immune responses, and (5) tumor responses via 18-fluorodeoxyglucose (18FDG) positron-emission tomography (PET) scans and chest CT scans. Repeat intrapleural Ad.IFN-ß doses were generally well tolerated. One patient with a pre-existing pericardial effusion developed pericardial tamponade. No MTD was reached. Intrapleural IFN-ß expression was detected in most patients after the first dose; however IFN-ß levels were markedly lower after the second vector dose delivered either 2 weeks (13 patients) or 1 week (4 patients) after the first dose. This lack of expression correlated with rapid Nab induction in nearly every patient. Strong humoral responses against known and unknown tumor antigens were induced in all patients. Several of the patients (4 of 13) had meaningful clinical responses (mixed and/or partial responses) as measured on pre-and post-Ad.IFN-ß infusion PET/CT imaging. In conclusion, repeat intrapleural Ad.IFN-ß doses were safe and induced immune and clinical responses in MPM and MPE patients. Rapid Nab development prevented effective gene transfer after the second dose even with a 7-day dose interval. A current trial delivers a second intrapleural Ad.IFN-ß vector 3 days after the first dose and in combination with chemotherapy.

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104 Delivery of Plasmid DNA-Encoding Cytokines for the Potential Treatment of Malignant Melanoma

Richard Heller[1,2], Bernadette Marrero[2], Adil Daud[3], Shawna Shirley[1], Kenneth Ugen[3]

[1]Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, VA; [2]Center for Molecular Delivery, University of South Florida, Tampa, FL; [3]Melanoma Clinical Research, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, San Francisco, CA

A critical aspect of gene transfer is effective delivery of the transgene to the appropriate target. Both biological (viral) and non-viral approaches have been evaluated. A drawback of the non-viral approach is ineffective expression of the transferred gene. This is typically related to the inefficient delivery of the plasmid DNA. Electrically mediated delivery (electroporation) of plasmid DNA is quickly being accepted as a viable approach to achieve effective delivery. The versatility inherent in this delivery system is an important reason for this growth. Our laboratory has examined the use of electroporation to delivery plasmid DNA encoding various cytokines as a potential therapy for melanoma. The plasmid is injected directly into the tumor followed by the administration of electroporation. We have evaluated several cytokines utilizing this approach, including IL-2, GM-CSF, IFNα, and IL-12. Previous reports from our laboratory have shown that delivery of a plasmid encoding IL-12 with electroporation results in an 80% cure rate of established B16.F10 tumors with minimal toxicity. These experiments provided the rationale for a Phase I proof-of-concept, first-in-man trial in patients with accessible subcutaneous metastases with melanoma. This first-in-human Phase I study demonstrated that this approach could induce responses in both treated and untreated lesions. Currently, we are evaluating the effectiveness of delivering a plasmid encoding IL-15 to induce an anti-tumor effect. We have shown in a mouse melanoma model (B16.F10) that delivery of pIL-15 directly to the established tumors leads to increased levels of IL-15 and tumor regression. We are also evaluating the effectiveness of delivering the plasmid peritumorally and in combination with other cytokines such as IL-12. The results from these studies demonstrate the feasibility and efficacy of in vivo electrogene delivery.

105 Exploiting Autophagy: Cross-Priming of Tumor-Specific T cells Against Short-Lived Proteins

Hong-Ming Hu, Yuhuan Li, Pui-yi Pang, Walter J. Urba

Earle A. Chiles Research Institute, Providence Portland Medical Center, Portland OR

In recent years, a number of cancer vaccines have been tested in Phase I to III clinical trials. These cancer vaccines were made of defined tumor-associated antigens (peptides, proteins, plasmid DNA, recombinant viruses) or undefined antigens (irradiated whole tumor cells, heat shock proteins, and dendritic cells [DC] loaded with tumor lysates or mRNA) or fused with tumor cells. However, the overall objective clinical response rate of vaccines tested in cancer patients was only 3.3% or less, as evaluated by conventional oncological criteria. No FDA-approved cancer vaccine is available so far. This disappointing outcome underscores the need for the development of novel vaccines. We believe that an effective vaccine needs to include multiple tumor-specific antigens and efficient cross-presentation of these tumor-specific antigens by dendritic cells. We recently showed that autophagy in tumor cells was essential for the efficient cross-presentation of tumor antigens, and autophaogosomes were efficient carriers to cross-prime antigen-specific CD8 T cells. During autophagy, tumor cells sequester damaged cytosolic proteins and superfluous organelles in a double-membrane structure, often referred to as an “autophagosome,” and deliver them to lysosomes for degradation. It is believed that most long-lived proteins are destroyed by autophagy-mediated lysosomal degradation, whereas short-lived proteins (SLiPs), including defective ribosomal products (DRiPs), are degraded by the proteosomes. We developed a novel strategy to increase the number of autophagosomes that favor cross-presentation of DRiPs from tumor cells by inducing autophagy with a proteasome inhibitor while stopping the maturation of autophagosomes. Autophagosomes could be increased in a variety of tumor cell lines, and the novel autophagosome-enriched DRiPs-containing blebs (DRibble) vaccine could treat established B16F10 melanoma and 3LL lewis lung tumor. Using HEK 293T cells that expressed ovalbumin (OVA) or gp100, we demonstrated that DRibbles sequestered model SLiP antigens and were highly efficient at stimulating antigen-specific naive CD8 and CD4 T cells in vitro and in vivo. Tumor-derived DRibble vaccines in combination with adjuvants were effective in treating mice bearing F10 tumor and 3LL tumors. Furthermore, we documented that dendritic cells use both caveolae- and clathrin-mediated endocytosis to up take DRibbles and the ERAD/proteasome/TAP1 dependent pathway to process and present antigens in DRibbles. Phase I/II NSCLC clinical trials based on the DRibble concept is underway, and we are continuing to investigate the basic mechanisms of the novel cross-presentation pathway.

106 Cluster Intradermal HPV DNA Vaccination Rapidly Induces E7-Specific CD8+ T Cell Immune Responses Leading to Therapeutic Antitumor Effects

Chien-Fu Hung

Departments of Pathology and Oncology, Johns Hopkins Medical Institutions, Baltimore, MD

Therapeutic human papillomavirus (HPV) vaccines targeting oncogenic protein E7 represent a potentially promising approach for the treatment of cervical cancer. Intradermal administration of HPV DNA vaccines via gene gun represents a feasible strategy to deliver DNA directly into the professional antigen-presenting cells (APCs) in the skin, thus improving DNA vaccine potency. We have previously demonstrated that DNA vaccines encoding HPV-16 E7 antigen linked to calreticulin (CRT) are capable of enhancing the E7-specific CD8+ T cell immune responses and antitumor effects against E7-expressing tumors in preclinical models. The clinical trial of therapeutic HPV DNA vaccines for patients with stage IB1 cervical cancer represents an excellent opportunity to assess the local antigen-specific immune responses in the tumor and draining lymph nodes (compared to systemic immune responses using PBMCs), since the standard care for stage IB1 cervical cancer requires radical hysterectomy and resection of the draining lymph nodes. However, it is ethically important to perform therapeutic HPV vaccinations within 1 month from the diagnosis until the tumor resection. Thus, it is essential to develop a vaccination regimen that can be completed before tumor resection (within a month). In the current study we hypothesize that the cluster (short-interval) intradermal CRT/E7 DNA vaccination will generate significant antigen-specific CD8+ T cell infiltrates in E7-expressing tumors in tumor-bearing mice, leading to an increase in apoptotic tumor cell death. We found that cluster intradermal CRT/E7 DNA vaccination is capable of rapidly generating a significant number of E7-specific CD8+ T cells, resulting in significant therapeutic antitumor effects in vaccinated mice. Thus, our study serves as an important foundation for future clinical trials in patients with stage IB1 cervical cancer.

107 Human Her-2 DNA Vaccines Containing Heterologous Rat Neu Sequence Effectively Overcome Her-2 Tolerance to Amplify Anti-Tumor Immunity

Jennifer B. Jacob[1], Elena Quaglino[2], Olga Radkevich-Brown[1], Federica Cavallo[2], Richard F. Jones[1], Amy Weise[1], Augusto Amici[3], Guido Forni[2], **Wei-Zen Wei**[1]

[1]Karmanos Cancer Institute, Wayne State University, Detroit, MI; [2]Molecular Biotechnology Center, Department of Clinical and Biological Sciences, University of Torino, Torino, Italy; [3]Department of Molecular, Cellular and Animal Biology, University of Camerino, Camerino, Italy

Her-2/neu DNA vaccination is a promising strategy for cancer control as we previously showed in Her-2/neu transgenic mice, but immune tolerance to this tumor-associated self antigen continues to be a challenge. In canine melanoma and several experimental autoimmune disease models, tolerance to self antigen can be overcome by immunization with heterologous (xenogeneic) antigens. To overcome immune tolerance to Her-2, DNA vaccines have been formulated to express both human Her-2 and heterologous rat Neu either in two separate plasmids or as chimeras that encode Her-2-neu fusion proteins, including pE2-NeuTM (HuRT), pE2-Neu500, or pNeu-E2TM (RHuT). Candidate vaccines were tested in Her-2 transgenic (Tg) mice of BALB/c (BALB), (BALB/cxC57BL/6) F1 (F1), and C57BL/6 (B6) background which exhibited decreasing immune reactivity to Her-2. Compared with wild-type pE2TM (Her-2 codons 1–687), the chimera pE2-NeuTM (Her-2 codons 1-390 fused to neu codons 391-688) induced elevated antibody and T cell responses in all test strains and tumors were rejected even in the most tolerant B6 Her-2 Tg mice. Although neu itself did not induce Her-2 binding antibodies, the presence of neu in the cocktail vaccine or chimera pE2-NeuTM enhanced antibody response to Her-2, showing immune stimulation by the heterologous neu. On the contrary, heterologous pNeuTM induced greater number of Her-2-specific IFN-γ producing T cells than self pE2TM, but only after repeated vaccination, suggesting a delayed but eventually more profound T cell activation by the cross-reactive neu epitopes. A reverse construct pNeu-E2TM containing neu codons 1–390 fused to Her-2 codons 391–687, or a modified pE2-Neu500TM with an abbreviated neu substitution, was less effective than pE2-NeuTM, indicating the requirement of specific Her-2 and neu sequences in immune activation. Rejection of Her-2-positive tumors resulted in epitope spreading to protect mice from Her-2-negative tumors. Therefore, combination of self human Her-2 and highly homologous, heterologous rat neu resulted in a potent vaccine regimen.

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108 Targeting MARCO Can Lead to Enhanced Dendritic Cell Motility and Anti-Tumor Activity

Norimasa Matsushita[1,2], Hiroshi Komine[1], Annabelle Grolleau-Julius[3], **Shari Pilon-Thomas**[1], James J. Mulé[1]

[1]Moffitt Comprehensive Cancer Center, Tampa, FL; [2]Department of Surgery, Tokyo Women's Medical University Hospital, Kawada-Cho, Shinjuku-Ku, Tokyo; [3]Department of Internal Medicine, University of Michigan Health System, Ann Arbor, MI

We reported that murine tumor lysate-pulsed dendritic cells (TP-DC) could elicit tumor-specific CD4+ and CD8+ T cells in vitro and in vivo. TP-DC treatments in vivo resulted in regression of established subcutaneous tumors and lung metastases. By gene array analysis, we reported a high level of expression of a novel member of the cell surface class A scavenger receptor family, MARCO, by murine TP-DC compared to unpulsed DC. MARCO is thought to play an important role in the immune response by mediating binding and phagocytosis but also in the formation of lamellipodia-like structures and dendritic processes. We have now examined the biologic and therapeutic implications of MARCO expressed by TP-DC. In vitro exposure of TP-DC to a monoclonal anti-MARCO antibody resulted in a morphologic change of rounding with disappearance of dendritic-like processes. TP-DC remained viable after anti-MARCO antibody treatment; had little, if any, change in production of IL-10, IL-12p70 and TNF-alpha; but demonstrated enhanced migratory capacity in a microchemotaxis assay. The use of a selective inhibitor showed MARCO expression to be linked to the p38 mitogen-activated protein kinase (MAPK) pathway. In vivo, anti-MARCO-antibody-treated TP-DC showed better trafficking from the skin injection site to lymph node, enhanced generation of tumor-reactive IFN-gamma producing T cells, and improved therapeutic efficacy against B16 melanoma; similar findings were obtained with DC generated from MARCO gene knock-out mice. These results, coupled with our finding that human monocyte-derived DC also express MARCO, could have important implications to human clinical DC vaccine trials.

109 Immunomodulatory Derivatives of Thalidomide (IMiD)-Induced Neutropenia Is Associated With PU.1 Downregulation and Myeloid Maturation Arrest

Rekha Pal[1], Sara A. Monaghan[2], Markus Y. Mapara[1], Peter Schafer[3], G. David Roodman[1], Lynn Moscinski[4], Alan List[5], **Suzanne Lentzsch**[1]

[1]Division of Hematology/Oncology, University of Pittsburgh Cancer Institute, Pittsburgh, PA; [2]Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA; [3]Biology Drug Discovery, Celgene Corporation, Summit, NJ; Departments of [4]Hematopathology and [5]Malignant Hematology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

Previous clinical trials have shown that immunomodulatory derivatives of thalidomide (IMiDs), including lenalidomide or pomalidomide, induce high response rates in patients with relapsed or refractory multiple myeloma. However, the most dose-limiting adverse effect of IMiDs is severe neutropenia. Here we investigated a possible mechanism of induction of neutropenia. In colony-forming assays using human CD34+ hematopoietic precursors treated with IMiDs, we observed a highly significant increase in myeloid progenitor formation at the expense of erythroid colonies. Treatment of hematopoietic progenitors with IMiDs showed an upregulation of CD33, a marker for immature myeloid cells. Further, in vitro treatment of CD34+ with IMiDs resulted in downregulation of the transcription factor PU.1. Bone marrow samples of patients treated with lenalidomide showed decreased PU.1 expression in myeloid cells. PU.1 gene knock-down models exhibit an increased granulopoiesis with impaired neutrophil maturation, resulting in an accumulation of promyelocytes. In accordance with that, Cathepsin G, as a promyelocytic marker, was highly upregulated under treatment with IMiDs measured by quantitative RT-PCR, western blot, and ELISA in vitro and also in patients treated with IMiDs, confirming the maturation arrest of neutrophil granulocytes. To confirm the maturational arrest of myeloid cells we evaluated sequential bone marrows from six patients treated with lenalidomide, comparing bone marrow features prior to treatment, during treatment, and at the time of grade 4 neutropenia. Marrows at the time of neutropenia showed an accumulation of myeloid precursors supporting impaired myeloid maturation. In five out of six patients, the myeloid:erythroid ratio increased with unchanged bone marrow cellularity. In summary, we show that IMiDs downregulate PU.1, a key transcription factor involved in granulocyte differentiation. The loss of PU.1 results in medullary accumulation of immature myeloid precursors with corresponding neutropenia in the peripheral blood. Our findings suggest that IMiDs do not induce neutropenia by marrow suppression but rather by transient block of maturation, which might be overcome by application of G-CSF.

110 The Dual Role of CD40 Molecules in Myeloid-Derived Suppressor Cell-Mediated T-Cell Suppression and T Regulatory Cell Activation

Ping-Ying Pan[1], Ge Ma[1], Kaare J. Weber[2], Junko J. Ozao[2], George Wang[1], Bingjiao Yin[1], Celia M. Divino[2], **Shu-Hsia Chen**[1,2]

Departments of [1]Gene and Cell Medicine and [2]Surgery, Mount Sinai School of Medicine, New York, NY

Previously we found that immune tolerance in mice with advanced malignancies was associated with the accumulation of myeloid-derived suppressor cells (MDSCs) and an increase in the number of T regulatory cells (Tregs). Moreover, MDSC induced tumor-specific Foxp3+ Tregs in tumor-bearing mice. Herein we show that CD40 expression on MDSC, which is significantly enhanced upon IFN-gama stimulation, is required for MDSC -mediated T-cell suppression and activation of tumor-specific Tregs. In a reconstitution model of MaFIA (cfms- Fas-induced apoptosis) mice, adoptively transferred Gr-1+CD115+ monocytic MDSCs derived from CD40 deficient mice, but not from wild-type mice, lost the capacity to induce T-cell tolerance and Treg development in vivo. Treatment with agonistic anti-CD40 antibodies inhibited the development of tumor-specific Tregs in tumor-bearing mice and significantly improved the therapeutic efficacy of combination therapy (IL-12 and 4-1BB activation) in the treatment of advanced tumors. Our findings suggest that CD40 is not only essential for MDSC-mediated immune suppression but is also required for tumor-specific Treg activation. Blockade of CD40 ligation may provide a new avenue to modulate both MDSC-mediated immune suppression and tumor-specific Treg activation in an immune-based cancer therapy.

111 Development of a Genetically Modifiable Humanized Mouse Model of Glioma Immunology

Ian F. Parney[1], Allan Nilson[1], Guido C. Gonzalez[1], Lung-Ji Chang[2], Allan B. Dietz[1]

[1]Mayo Clinic College of Medicine, Rochester, MN; [2]University of Florida, Gainesville, FL

Patients diagnosed with malignant gliomas such as glioblastoma multiforme (GBM) face a dismal prognosis despite aggressive treatment. Immunotherapy is a promising therapeutic strategy that has been hampered by tumor-mediated immunosuppression. GBM cells are directly immunosuppressive through several mechanisms (B7-H1, TGF-ß2, PGE2,and IL-6). GBM tumors are also heavily infiltrated with immunosuppressive monocytic cells. Finally, GBM patients are systemically immunosuppressed with decreased T cell responsiveness; increased regulatory T cells (Treg), despite overall reductions in CD4+ cells; increased myeloid-derived suppressor cells (MDSC); and decreased immature dendritic cells (DC). Developing effective immunotherapies would benefit from a manipulatable animal model that closely replicated human glioma pathology and immunity. NOD/scid/IL2Rγ-null (NSG) mice can be robustly reconstituted with a full spectrum of human myeloid and lymphoid cells after tail vein injection with human CD34+ hematopoietic stem cells (HSC). To facilitate this, we have developed techniques to purify and expand CD34+ HSC from discarded leukapheresis tubing from normal donors following G-CSF-mediated stem cell mobilization. We have also developed human GBM stem cell lines that closely replicate human GBM tumors when implanted intracranially in NSG mice. Both the GBM stem cell lines and the HSC are potentially amenable to gene transfer using lentiviral vectors. Experiments are ongoing to determine the effect of intracranial human GBM tumors on human myeloid and lymphoid reconstitution in NSG mice receiving HSC injections. We are specifically testing the hypothesis that HSC-reconstituted NSG mice with human GBM tumors will develop signs of systemic immunosuppression similar to patients with GBM. Comparison will be made to non-tumor-bearing mice reconstituted with HSC. The effects of HSC reconstitution on GBM tumor growth are also being evaluated. Finally, our ability to stably transduce the GBM cells and HSC cells with a reporter gene (Green Fluorescent Protein) using a lentiviral vector prior to injection into the mice and the longevity of transgene expression in vivo is being assessed.

112 Secreted gp96-Ig in Innate Immune Activation and Cellular Vaccine Development

Eckhard R. Podack, Natasa Strbo, Satoshi Oizumi, Taylor Schreiber

Department of Microbiology and Immunology, University of Miami, Leonard Miller School of Medicine, Miami, FL

Necrotic cell death can be caused by trauma or by infectious agents. It is critical for the immune system to distinguish between these two causes and to initiate an immune response to the infectious agent. While bacterial components are recognized by TLRs, there is no simple way for recognition of viral protein antigens and generation of a cytotoxic response. We provide evidence that DC, as part of the natural immune system, samples peptides chaperoned by heat shock proteins for presentation to CD8 T cells. Foreign peptides chaperoned by heat shock proteins and picked up via heatshock protein receptors cause DC activation, recruit and activate NK cells, and powerfully cross-prime CD8 CTL without CD4 help. MHC class I-mediated cross-priming of CD8 T cells by APCs is critical for CTL-based immunity to viral infections and tumors and is required for the development of vaccines stimulating cytotoxicity and the subsequent development of immunological memory. We have shown previously that tumor-secreted heat shock protein gp96-Ig-chaperoned peptides cross-prime CD8 CTL that are specific for genuine tumor Ags and for the surrogate Ag OVA. Tumor-secreted heat shock protein gp96-chaperoned peptides enhance the efficiency of Ag cross-priming of CD8 CTL by several million-fold over the cross-priming activity of unchaperoned protein alone. gp96 also acts as adjuvant for cross-priming by unchaperoned proteins, but in this capacity gp96 is 1,000-fold less active than as a peptide chaperone. Mechanistically, the in situ secretion of gp96-Ig by transfected tumor cells recruits and activates dendritic cells and NK cells to the site of gp96 release and promotes CD8 CTL expansion locally. gp96-mediated cross-priming of CD8 T cells requires B7.1/2 costimulation but proceeds unimpeded in lymph node-deficient mice, in the absence of NKT and CD4 cells and without CD40L. Gp96-driven MHC I cross-priming of CD8 CTL in the absence of lymph nodes provides a novel mechanism for local, tissue-based NK and CTL generation at the site of gp96 release. This pathway may constitute a critically important early detection and rapid response mechanism that is operative in parenchymal tissues for effective defense against tissue damaging antigenic agents. Immunotherapy for NSCLC-patients using an allogeneic NSCLC cell line secreting gp96-Ig is in progress in a Phase I study and shows good immunogenicity and safety. Studies using SIV-antigen transfected, gp96-Ig-secreting 293 cells as vaccine in NHP demonstrate powerful, multifunctional mucosal CTL immunity to multiple SIV epitopes.

113 mda-7/IL-24: Broad-Spectrum Gene Therapeutic for Localized and Disseminated Cancers

Devanand Sarkar[1,2,3], Zhao-Zhong Su[1], Adly Yacoub[4], Igor Dmitriev[7,8], Steven Grant[5], Paul Dent[6], David T. Curiel[8], **Paul B. Fisher**[1,2,3]

[1]Department of Human and Molecular Genetics, [2]Institute of Molecular Medicine, [3]Massey Cancer Center, Departments of [4]Radiation Oncology, [5]Medicine, and [6]Biochemistry and Molecular Biology, Virginia Commonwealth University, School of Medicine, Richmond, VA; [7]Department of General Surgery/Oncology and the [8]Division of Human Gene Therapy, Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL

Cancer cells frequently display abnormalities in differentiation, which are reversible in particular cancers. Our research exploits this property of tumor cells reversing the cancer phenotype by inducing terminal differentiation—differentiation therapy of cancer. Exposure of human melanoma cells to interferon and mezerein reverts cancer cells to a more normal state and causes irreversible growth arrest and terminal cell differentiation. Using this model system combined with subtraction hybridization we cloned melanoma differentiation-associated gene-7 (mda-7), which is a new member of the interleukin (IL)-10 gene family, designated IL-24. mda-7/IL-24 displays several exceptional properties that contribute to its potential as an effective cancer gene therapy. It selectively induces growth suppression and apoptosis in cancer cells of diverse origin, does not harm normal cells, inhibits tumor development and progression in vivo in human tumor xenograft models, induces potent antitumor “bystander” effects, inhibits tumor angiogenesis, enhances antitumor effects of radiation, chemotherapy and monoclonal antibody therapy, and modulates immune functions. mda-7/IL-24 selectively induces endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) in tumor cells. In the in vivo setting, it is likely that the combined actions of this intriguing cytokine contribute to its profound anti-cancer activity. mda-7/IL-24 has entered the clinic, recapitulating many of the effects apparent in cell culture and in animal models, namely induction of tumor cell-specific apoptosis, “bystander” antitumor activity, and immune modulation. In patients with advanced cancers in a Phase I clinical trial, multiple intratumoral injections with a replication incompetent adenovirus expressing mda-7/IL-24, Ad.mda-7 (INGN 241) were safe and provoked significant clinical activity. Future strategies employing conditionally replicating and tropism-modified Ads to deliver mda-7/IL-24 and combining this therapy with additional therapeutic agents offer promise for further enhancing the therapeutic benefit of this novel cytokine.

114 Modulation of T Cell Activation by Malignant Melanoma-Initiating Cells

Tobias Schatton[1], Ute Schütte[1], Qian Zhan[2], André Hörning[1], George F. Murphy[2], **Markus H. Frank**[1,3]

[1]Transplantation Research Center, Children's Hospital Boston and Brigham and Women's Hospital, Boston, MA; [2]Department of Pathology, Brigham and Women's Hospital, Boston, MA; [3]Department of Dermatology, Brigham and Women's Hospital, Boston, MA

Experimental tumor models and clinical findings in human patients suggest a negative correlation between degrees of host immunocompetence and rates of tumor initiation and growth. Moreover, even highly immunogenic cancers such as malignant melanoma are capable of inexorable growth despite the presence of robust host immunity. These findings raise the possibility that under conditions of relatively intact immunity, only a restricted minority of malignant cells, i.e., cancer stem cells (CSC), might possess the phenotypic and functional characteristics to evade host immunosurveillance and immune-mediated rejection. Here we provide initial evidence supporting this intriguing hypothesis by demonstrating that ABCB5+ malignant melanoma-initiating cells (MMIC), a recently identified cancer subpopulation enriched for CSC, possess the capacity to preferentially inhibit interleukin (IL)-2-dependent T cell activation and to support, in a B7.2-dependent manner, regulatory T (Treg) cell induction. ABCB5+ MMIC exhibited significantly reduced major histocompatibility complex (MHC) class I expression and aberrant positivity for MHC class II. In addition, ABCB5+ subpopulations selectively expressed the costimulatory ligand B7.2 in both established melanoma xenografts and clinical tumor specimens in vivo. In vitro, ABCB5+ melanoma cells inhibited mitogen-dependent human peripheral blood mononuclear cell (PBMC) proliferation and IL-2 production more efficiently than ABCB5- tumor bulk populations. Moreover, coculture with ABCB5+ melanoma subsets significantly increased, in a B7.2 signalling-dependent manner, CD4+CD25+FoxP3+ Treg cell abundance and IL-10 production by mitogen-activated PBMC. Our findings identify novel T cell-modulatory functions of ABCB5+ melanoma subpopulations and point to specific roles for MMIC in the evasion of host immunosurveillance and in cancer immunotherapeutic resistance.

115 Biology and Therapy of High-Risk Neuroblastoma

Robert C. Seeger[1], Yves A. DeClerck[1], Shahab Asgharzadeh[1], William A. Weiss[2], Katherine K. Matthay[2], Judith G. Villablanca[1], Hollie Jackson[1], Heike Daldrup-Link[2], Randall Hawkins[2], Hiroyuki Shimada[1], Michael Sheard[1], Nino Keshelava[1], Susan Groshen[1], Richard Sposto[1]

[1]Departments of Pediatrics, Pathology/Laboratory Medicine, and Radiology, Childrens Hospital Los Angeles and Keck School of Medicine, University of Southern California, Los Angeles, CA; [2]Departments of Pediatrics, Neurology, and Radiology, University of California, San Francisco, CA

Neuroblastoma is the most common extracranial solid tumor of childhood, and 45% of patients have high-risk, metastatic disease. Over the past 20 years, long-term survival has improved to 40% with increasing intensity of non-specific cytotoxic induction and consolidation therapy and with biotherapy of minimal residual disease after consolidation. We hypothesize that further improvement in survival for newly diagnosed patients as well as for established patients with refractory or recurrent disease will result from new combinatorial therapies that target critical pathways of tumor and host cells that promote neuroblastoma growth and survival in primary and metastatic sites. Our Specific Aims are as follows: (1) perform biologic research to identify and further understand molecules and pathways of tumor and microenvironment cells that are responsible for neuroblastoma growth; (2) perform pre-clinical therapeutic research with drugs and biologics to develop effective strategies against neuroblastoma; (3) perform early-phase clinical trials of combinatorial strategies targeting molecules and pathways of tumor and microenvironment cells to determine appropriate dose and schedule, pharmacology and pharmacodynamics, and, within the context of such trials, anti-tumor activity. Project 1: The bone marrow microenvironment is investigated with emphasis on the role of IL-6 and STAT3 activation in bone marrow and bone metastasis and on identifying effective drugs and biologics that target this pathway. Project 2: Immunotherapy strategies maximize natural killer (NK) cell activity with tumor cell-targeting antibodies and agents that modify the tumor microenvironment milieu to minimize NK suppressive effects of monocytes/macrophages producing IL-6 and TGFß1. Project 3: Small molecules, including PI3K, PI3K+mTOR, and Aurora Kinase A inhibitors that result in destabilization and degradation the MYCN protein, are investigated for their anti-tumor cell activity as well as their effects upon microenvironment cells. Project 4: New strategies developed in our laboratories are tested in Phase I and II trials by the New Approaches to Neuroblastoma Therapy (NANT) consortium (www.nant.org), which includes 15 pediatric oncology institutions across the United States and in Canada. Critical pathways of tumor and host cells responsible for neuroblastoma growth are identified and validated, and strategies using drugs and biologics to target these pathways are developed with pre-clinical models. Delivery of the new strategies to patients is accomplished by our early phase (NANT) clinical trials consortium.

116 Genital Mucosal Immune Responses Are More Predictive of Lesion Regression in Preinvasive HPV Neoplasia Than Systemic Virus-Specific Response

Cornelia L. Trimble[1], Christopher Thoburn[1], Shiwen Peng[1], Nicole C. Hanson[3], Jodie Tassello[3], Denise Frosina[3], Ferdynand Kos[1], Rachael A. Clark[2], Achim A. Jungbluth[3]

[1]Johns Hopkins University School of Medicine, Baltimore, MD; [2]Department of Dermatology, Harvard Skin Disease Research Center, Brigham and Womens' Hospital, Boston, MA; [3]Ludwig Institute for Cancer Research at Memorial Sloan-Kettering Cancer Center, New York, NY

Essentially all cervical squamous cell cancers and their precursor lesion, high-grade cervical intraepithelial neoplasia (CIN2/3), are caused by persistent human papillomavirus (HPV) infection. Both are associated with viral integration into the host genome and subsequent expression of two viral oncoproteins, E6 and E7, which are functionally obligate for disease initiation and persistence. However, not all CIN2/3 lesions progress to cancer. In a brief, 15-week observational study protocol monitoring subjects from biopsy diagnosis (t0) to definitive therapy (cervical conization at twk15), we reported previously that 25% of CIN2/3 lesions associated with HPV16, the genotype most commonly associated with disease, underwent complete regression. HPV16-specific T cell responses measured in peripheral blood obtained at the time of study entry and at the time of conization were marginally detectable directly ex vivo and did not correlate with lesion regression. Here we present immunologic studies of the lesional mucosa: memory T cells accumulate in dysplastic mucosa, and spectratyping provides strong evidence that these populations often reflect clonal expansions. The degree of lesional intraepithelial CD8+ infiltration at the time of study entry was predictive of regression by week 15. In contrast, in lesions that failed to regress, immune cell infiltrates increased in individual subjects but were restricted to the stromal compartment, while intraepithelial CD8+ infiltrates remained minimal. Mechanisms by which preinvasive HPV-associated epithelial lesions are likely to mediate immune evasion, well before development of an invasive phenotype, will be discussed.

117 Helper Activity of NK Cells and NK Cell-Related Factors in DC-Mediated Induction of CCR5+CXCR3+ Melanoma-Specific CTLs

Payal Watchmaker[1], Howard Edington[1], Robbie Mailliard[1], Erik Berk[1], Ravikumar Muthuswamy[1], Julie A. Urban[1], Stergios Moschos[2], Michael T. Lotze[1], John M. Kirkwood[2], **Pawel Kalinski**[1]

Departments of [1]Surgery and [2]Medicine, University of Pittsburgh Cancer Institute, Pittsburgh, PA

The ability of cancer vaccines to induce tumor-specific CD8+ T cells in the blood of melanoma patients has been shown to poorly correlate with the clinical effectiveness of vaccination. Here, we report that although antigens presented by different types of mature dendritic cells (DCs) are similarly effective in inducing CD8+ T cell expansion, the acquisition of CTL function and melanoma-directing chemokine receptors CCR5+ and CXCR3+ requires antigen presentation by “non-exhausted” (or type-1-polarized) DCs, induced by NK cells or NK cell-related cytokines. Both “standard” DCs (matured in the presence of PGE2) and type-1-polarized DCs are similarly effective in inducing CD8+ T cell expansion and acquisition of CD45R0+IL-7R+IL-15R+ phenotype. However, Granzyme B expression, acquisition of CTL activity, and peripheral tissue-type chemokine responsiveness are features exclusively exhibited by the CD8+ T cells induced by type-1-polarized DCs. This advantage of type 1-polarized DCs was observed in polyclonally activated naïve and memory CD8+ T cells, as well as in blood-isolated melanoma-specific CTL precursors and melanoma-isolated TILs with defective CTL function. Our data help to explain the dissociation between the ability of cancer vaccines to induce high numbers of tumor-specific CD8+ T cells in the blood of cancer patients and their ability to promote clinical responses, providing for new strategies of cancer immunotherapy.

118 Molecular Mechanisms of Immune Suppression and Biologic Significance

Theresa L. Whiteside, Magis Mandapathil, Malgorzata Czystowska, Laura Strauss, Bill Gooding, Elieser Gorelik

University of Pittsburgh Cancer Institute

Regulatory T cells (Treg) are crucial for maintaining tolerance to self and thus preventing autoimmune diseases and allograft rejections. We and others have reported increased numbers of Treg at the tumor site and in the circulation of patients with cancer. Both naturally occurring (nTreg: CD4+CD25highFOXP3+) and inducible (Tr1: CD4+CD25negTGF-ß1+IL-10+) Tregs up-regulate their functions in patients with cancer, and Treg-mediated suppression of anti-tumor immune effector cells promotes tumor escape. However, the mechanisms used by Treg to mediate suppression are not yet defined. Also, the prognostic significance of the increased Treg frequency in cancer is being debated. Our phenotypic and functional studies with human CD4+CD25highFOXP3 Treg isolated from blood or tumor tissues of patients with head and neck cancer (HNC) have identified the following molecular mechanisms of suppression: (a) IL-10- and/or TGF-ß1-mediated inhibition of autologous responder cell (RC) proliferation and/or cytokine production; (b) adenosine-mediated inhibition of multiple effector cell functions by ectonucleotidase-expressing CD39+ and CD73+ nTreg or Tr1 cells; (c) the Fas-FasL pathway-mediated death of CD8+ responder T cells by activated Treg; and (d) the involvement of cytotoxins (granzymes A and B and perforin) in suppression mediated by Treg. Using in vitro models in which single-cell sorted Treg are co-cultured with autologous RC, we show that human Treg can utilize all of the above pathways to contract immune responses and that CD4+CD25highFOXP3+ Treg and CD4+CD25neg RC reciprocally regulate death and expansion arrest by differentially utilizing these pathways, depending on the microenvironmental context. In HNC patients Treg generation is promoted at the tumor site. Treg expand during disease progression and following cancer therapy in patients with no evident disease (NED). The persistent presence of increased numbers of Treg mediating high levels of suppression in these patients suggests that the normalization of Treg in cancer is not readily achievable and remains a major therapeutic goal in the future.

119 Real-Time Singlet Oxygen Monitor for Photodynamic Therapy

Steven J. Davis[1], Seonkyung Lee[1], Tayyaba Hasan[2]

[1]Physical Sciences, Inc.; [2]Massachusetts General Hospital

Photodynamic therapy (PDT) is a relatively new, rapidly developing, and promising modality for cancer treatment. PDT uses certain compounds known as photosensitizers (PSs) that are preferentially retained in malignant tumors. With visible light, the photosensitizers initiate a reaction that selectively kills the malignant cells to which they are attached. PDT is being used in clinical trials for bladder, brain, skin, and other cancers. PDT is also being applied to important areas outside of cancer treatment including age-related macular degeneration and actinic keratosis, a pre-cancerous skin condition. There is considerable evidence that singlet molecular oxygen, O2(a1Δ), produced by energy transfer from optically excited PSs is the active species in cancer cell or endothelial cell necrosis. Despite the general acceptance of this role of O2(a1Δ) in PDT, there have been limited demonstrations of its importance in vivo. If O2(a1Δ) is indeed the critical species that determines PDT efficacy, a device that is conducive to online measurement of O2(a1Δ) in vivo could, in principle, provide the critical parameter in PDT dosimetry and the potential of individualized therapeutic design. In this poster we describe the development and testing of instruments to measure singlet molecular oxygen produced by the photodynamic process. Singlet oxygen is an active species in photodynamic therapy, and we are developing instruments for PDT researchers with the goal of a real-time dosimeter for singlet oxygen. Our optically-based method uses the weak but unique spectral signature from the O2(a-X) phosphorescence at 1.27 μm. Results of in vitro tests to characterize the sensors and preliminary in vivo results will be presented.

120 A Small Animal Radiation Research Platform With Novel Radiobiological Applications

E. Ford, P. Kazanzides, E. Armour, T. McNutt, E. Tryggestad, J. Wong

The Johns Hopkins University, Baltimore, MD

The irradiation technology employed by experimental radiobiology lags far behind the precision radiation delivery technology commonly available in the clinical setting. A more precise laboratory irradiation technology would enable new class of radiobiological experiments. We have designed and constructed a Small Animal Radiation Research Platform (SARRP) which is capable of on-board CT-guided radiation treatment of rodents. The unit is semi-portable (3 feet by 4 feet by 6 feet). X-ray beams are delivered via a constant voltage x-ray source with dual-focal spots (0.4 mm and 3.0 mm) in either imaging mode (50–100 kVp) or treatment mode (225 kVp). Cone-beam CT is acquired with a flat panel amorphous silicon detector. Reconstructed voxel resolutions of 0.55 by 0.55 by 0.55 mm3 are acquired with less than 1 cGy in 2 minutes. Radiation beams can be collimated from 0.5 mm in diameter to (60 by 60) mm2 with 1 cm depth outputs ranging from 100 (small field) to 375 cGy/min (largest field). Robotic translate/rotate stages are used to position the animal. Conformal dose distributions are delivered using a combination of gantry and robotic stage motion. Treatment planning is accomplished with Monte Carlo dose calculations. Overall system accuracy has been measured at 0.2 mm. Immunohistochemical validation on tissue demonstrates that the beam can be located to the intended target with an accuracy of 0.68 mm. The ability of our system to focally irradiate a specific anatomic region or target in a laboratory animal opens new avenues of pre-clinical research. Examples include the study of localized radiation and neurogenesis in the brain, where we have demonstrated that targeted millimeter-scale beams can deplete proliferating neural progenitor cells over long time scales. Validation of novel molecular imaging agents in the radiation setting is another application, as demonstrated by our studies of peripheral benzodiazepine receptor ligand to image early inflammatory response after localized irradiation of the lung. Other applications being explored in our collaborations include vaccine therapies against pancreatic or prostate cancers in combination with local radiation and the use of magnetically-heated iron nanoparticles with radiation. A wide variety of novel experimental radiobiological applications are possible with this technology.

121 Molecular Response and Imaging-Based Combination Strategies for Optimal PDT

Tayyaba Hasan[1], Stephen P. Pereira[2], Brian W. Pogue[3], Edward V. Maytin[4]

[1]Massachusetts General Hospital and Harvard Medical School, Boston; [2]University College London Medical School, London UK; [3]Dartmouth College, Hanover, NH; [4]Cleveland Clinic Foundation, Cleveland OH

The underlying hypothesis of this program is that the complexity of cancer dictates that compared to single modalities, combination therapies that are mechanistically independent and directed at non-overlapping molecular targets will additively or synergistically enhance treatment outcome. Our program proposes new photodynamic therapy (PDT)-based combination treatments for pancreato-biliary tract cancer (PCBC) in an approach we term Combination Photodynamic Biologic Therapy (CPBT). PDT is a photochemistry-based modality approved for a number of cancer and non-cancer pathologies and has shown promise for the treatment of PCBC, where other approaches have failed. This work builds on recent advances in the understanding of cancer biology and mechanisms of current and emerging therapies, as well as the enormous progress made in imaging technologies. Strategically, we are exploring these treatment response-enhancing CPBTs by administering a second treatment specifically tailored to a particular molecular response elicited by PDT. This work is being conducted simultaneously in clinical and pre-clinical settings. In clinical studies, we are evaluating the ability of PDT to improve the survival and quality of life for patients with PCBC. In parallel, we are conducting preclinical studies to target molecular responses that are elicited by PDT to design and optimize new clinically relevant CPBTs, such as combining PDT with Erbitux-based inhibition of the epidermal growth factor receptor. Leveraging the multidisciplinary nature of this program, we are utilizing advanced optical imaging platforms to quantitatively monitor molecular responses to treatment, such as vascular endothelial growth factor (VEGF). Additionally, we will use optical imaging as a tool for light and photosensitizer dosimetry, which will improve treatment planning and therapeutic outcome. We envision that these longitudinal imaging studies will be integrated into the development of molecular-based combination therapies for standard clinical procedures. The program is supported by one administrative and two scientific cores, which provide a central platform access for meetings, imaging, and other technological advances and the transfer of developed technology to industry. It is anticipated that such rational, mechanism-based treatments will significantly benefit patient survival and, combined with real-time imaging to monitor tumor progression and treatment response, provide patient-specific treatments. This research will have significant impact on the clinical outcomes for patients with PCBCs, two deadly diseases which currently have few treatment options.

122 Delivery of Plasmid DNA Encoding Cytokines for the Potential Treatment of Malignant Melanoma

Richard Heller[1,2], Bernadette Marrero[2], Adil Daud[3], Shawna Shirley[1], and Kenneth Ugen[3]

[1]Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, VA; [2]Center for Molecular Delivery, University of South Florida, Tampa; [3]Melanoma Clinical Research, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco

A critical aspect of gene transfer is effective delivery of the transgene to the appropriate target. Both biological (viral) and non-viral approaches have been evaluated. A drawback of non-viral is ineffective expression of the transferred gene. This is typically related to the inefficient delivery of the plasmid DNA. Electrically mediated delivery (electroporation) of plasmid DNA is quickly being accepted as a viable approach to achieve effective delivery. The versatility inherent in this delivery system is an important reason for this growth. Our laboratory has examined the use of electroporation to delivery plasmid DNA encoding various cytokines as a potential therapy for melanoma. The plasmid is injected directly into the tumor followed by the administration of electroporation. We have evaluated several cytokines utilizing this approach including IL-2, GM-CSF, IFNα, and IL-12. Previous reports from our laboratory have shown that delivery of a plasmid encoding IL-12 with electroporation results in an 80% cure rate of established B16.F10 tumors with minimal toxicity. These experiments provided the rationale for a Phase I proof-of-concept, first-in-man trial in patients with accessible subcutaneous metastases with melanoma. This first-in-human Phase I study demonstrated that this approach could induce responses in both treated and untreated lesions. Currently, we are evaluating the effectiveness of delivering a plasmid encoding IL-15 to induce an anti-tumor effect. We have shown in a mouse melanoma model (B16.F10) that delivery of pIL-15 directly to the established tumors leads to increased levels of IL-15 and tumor regression. We are also evaluating the effectiveness of delivering the plasmid peritumorally and in combination with other cytokines such as IL-12. The results from these studies demonstrate the feasibility and efficacy of in vivo electrogene delivery.

123 Block Copolypeptide Vesicles for Drug Delivery

Daniel T. Kamei

University of California, Los Angeles

The use of drug delivery vehicles has helped in overcoming some of the obstacles associated with delivering a naked drug. In our collaborative research with Professor Tim Deming's laboratory at the University of California, Los Angeles, we have been investigating novel copolypeptide vesicles comprising polyarginine and polyleucine segments as potential drug carriers. Specifically, we recently demonstrated through in vitro experiments that these vesicles can transport dye-labeled dextran into multiple cell lines without being toxic. These vesicles were formed from a block copolypeptide comprised of 60 arginine residues followed by 20 leucine residues. These vesicles are stable in media, can be processed to different sizes, and can be prepared in large quantities. In contrast to conjugating a polyarginine peptide to an existing vesicle, the polyarginine segment of these block copolypeptides directs both vesicular assembly and intracellular delivery. To further characterize the vesicles as drug delivery vehicles, the ability to control the size of the vesicles was investigated by exploring different methods to extrude the vesicles. The resultant size distributions from different extrusion methods were analyzed by dynamic light scattering, and the cytotoxicity of vesicles of different sizes was investigated to see whether vesicle size affects cytotoxicity. We are currently extending these characterization studies to vesicles formed from polypeptides of varying lengths.

124 The Nanoparticle-Based Bio-Barcode Assay Re-Defines 'Undetectable' PSA and Biochemical Recurrence Following Radical Prostatectomy

Chad A. Mirkin, C. Shad Thaxton

Northwestern University

Prostate cancer (PCa) is the most common cancer among American men and the third leading cause of cancer mortality. Importantly, up to 40% of patients who undergo PCa treatment in the form of radical prostatectomy go on to PCa recurrence. Although controversial in the setting of PCa screening, prostate specific antigen (PSA) is a clinically validated and powerful marker for diagnosing PCa recurrence. Currently, a post-operative PSA value rising from levels undetectable with conventional PSA assays (<0.1 ng/mL) to greater than 0.2 ng/mL is diagnostic of PCa recurrence. Clinical data demonstrate that early adjuvant and/or salvage treatment for PCa and intervention at the earliest sign of PSA recurrence results in an increase in PCa specific and overall survival. Thereby, we hypothesized that measuring serum PSA values at levels less than 0.1 ng/mL would enable us to define and quantify kinetic PSA parameters in the sub-0.1 ng/mL PSA range that: (1) are diagnostic of PCa cure; (2) define early PCa recurrence with a significant lead time with regard to conventional assays; and (3) allow for the quantitative evaluation of PSA profiles in patients receiving adjuvant and/or salvage treatment. Utilizing a nanotechnology-enabled and ultra-sensitive PSA immunoassay developed in the Mirkin laboratory with over 300 times the sensitivity of the conventional tests (limit of detection=0.3 fg/mL), we performed both a pre-clinical (n=18 patients) and large retrospective (n=417 patients to date) analysis to directly compare the PSA results obtained from the two methods and test our hypotheses. Importantly, data from each of these studies demonstrates that screening the serum of men with the ultra-sensitive PSA immunoassay enables: (1) the measurement of PSA values consistent with disease cure; (2) a diagnostic lead time of PCa recurrence; and (3) quantitative assessment of the PSA response to adjuvant and/or salvage treatments. Overall, these data argue for a prospective trial comparing the results of the ultra-sensitive PSA immunoassay and conventional assays in order to fully grasp the clinical utility of this unique and powerful technology.

125 Development of "See and Treat" Approaches in Photodynamic Therapy Based on the New Photosensitizer HPPH

Ravindra K. Pandey[1], William White[1], Manivannan Ethirajan[1], Nadine James[1], Yihui Chen[1], Joseph Spernyak[2], Carrie Batt[1], Munawwar Sajjad[3], **Barbara W. Henderson**[1]

[1]Cell Stress Biology Department and [2]MRI Facility, Roswell Park Cancer Institute, Buffalo, NY; [3]Department of Nuclear Medicine, State University of New York, Buffalo

Photodynamic therapy (PDT) is a modality for the local treatment of early and advanced solid tumors, which entails the activation by light of a tumor-localized photosensitize. Tumor-avid photosensitizers such as HPPH (2-1[hexyloxyethyl]-2-deviny lpyropheophorbide-a), developed at the PDT Center at Roswell Park Cancer Institute, show great promise in Phase I/II clinical trials. HPPH has been licensed for commercial development. One of the challenges in certain cancer therapies is the identification of tumor margins, especially in cancers with field disease characteristics or highly infiltrating cancers. In such cases the real-time imaging of the tumor to be removed and/or treated would be of great benefit. We are exploiting the tumor avidity of HPPH to develop a "See and Treat" approach through the design of bi-and multifunctional agents that combine the properties of the photosensitizer for PDT with those of imaging by fluorescence (optical imaging), magnetic resonance imaging (MRI), and/or positron emission tomography (PET). HPPH was conjugated with a non-tumor-specific cyanine dye-based fluorophore. The cyanine dye allows visualization of the tumor, while the presence of the photosensitizer allows subsequent tumor ablation. The conjugate showed significant tumor imaging capability in whole-body imaging of mice carrying subcutaneous transplantable tumors or orthotopic brain tumors. With imaging occurring 24 hours after administration, tumor visualization was successful at injected doses 8–10-fold less than the therapeutic dose for PDT. Under similar conditions, the free-cyanine dye showed significantly lower tumor-imaging capability. Following a similar approach, HPPH linked to 3[Gd(III)DTPA] resulted in a novel agent successfully combining tumor MR imaging and PDT properties when tested in mice and rats. Tumor-avid HPPH was also used as the core component for the design and synthesis of the bifunctional PET/PDT agent 3-devinyl-3-(1'-iodobenzyloxy)ethyl pyropheophorbide-a. When tested in a small animal scanner, optimal images were obtained in mice from the primary tumor sites as well as distant metastases at 48 hours after administration of the agent, while PDT efficacy was preserved. Initial testing indicated no or minimal prolonged skin photosensitization. Having established preclinical efficacy and following the translational paths established at RPCI, these novel compounds are currently undergoing preclinical toxicology.

126 Designing Hydrogel Materials That Aid Parenteral Delivery of Therapeutics

Joel Schneider[1], Monica Branco[1,2]

Departments of [1]Chemistry and Biochemistry and [2]Chemical Engineering, University of Delaware

Hydrogels are heavily hydrated materials that are finding use as extracellular matrix substitutes for tissue engineering and as vehicles for the delivery of therapeutics (e.g., small molecules, biomolecules, and cells). We are developing hydrogels that aid the parenteral delivery of small protein and antibody therapeutics. The exquisite specificity enjoyed by this class of drugs is often accompanied by limitations due to their susceptibility towards degradation during storage and upon administration. We have designed self-assembling peptides that undergo sol-gel phase transitions in response to biological media enabling the direct encapsulation and subsequent shear-thin delivery of protein-based therapeutics. Since hydrogelation is governed by self-assembly, the bulk material properties of these peptide hydrogels can be modulated by the molecular design of the constitutive monomeric peptides. Thus, the mechanical rigidity, crosslink density, mesh size, and electrostatic properties of the gel can be varied to fine-tune the release profiles of encapsulated therapeutics. This study focuses on determining the mass transport properties of model proteins from self-assembled peptide hydrogels before and after delivery via syringe. Proteins of varying molecular weights and pIs were studied to assess the effect of hydrogel charge density and mesh size on release. Depending on the formulation of the peptide hydrogel, the size and electrostatic nature of the encapsulated therapeutic, bulk protein release from the hydrogel can be varied from days to months.

127 Image-Guided Adaptive Radiotherapy for Head and Neck Cancer

David Schwartz, Lei Dong

The University of Texas M. D. Anderson Cancer Center

We are prospectively testing clinical deployment of an adaptive radiotherapy (ART) procedure using daily volumetric image guidance (IGRT) and serial replanning during oropharyngeal IMRT in a Phase I trial. Deformable image registration software permits mapping of structures and CTVs defined in the original plan to daily CT imaging. We have compared four planning scenarios: (1) the original IMRT plan aligned to the marked isocenter (BB); (2) the original plan aligned according to daily bone alignment (IGRT); (3) IGRT with one adaptive replan (ART1); and (4) IGRT with two adaptive replans (ART2). Baseline IMRT plans conformed to our current practice standards (3mm PTV), but all subsequent replanning used no PTV margins. We performed dosimetric evaluations by deformably mapping daily doses retrospectively back to the original plan. Eighteen patients have enrolled. All patients received at least one replan (ART1), and four patients received two replans (ART2). Large anatomic changes were seen on Mondays. The median trigger point for first adaptive replan was the 17th treatment fraction (range: 2 to 28), at which point the bilateral parotid volumes had shrunk by 17%, and combined CTVs had shrunk by 5%. For ART2 patients, median trigger point for the first replan was the 9th fraction, and for 2nd replan, the 20th fraction (range: 11 to 23), at which point bilateral parotid volumes and CTVs had shrunk by 20% and 8%, respectively. In one ART2 patient, GTV increased 50% between planning CT and first day of treatment, resulting in immediate replanning (2nd fraction). For 13 fully evaluable patients, all planning scenarios provided adequate target coverage, indicating that current standard non-IGRT practice (3mm PTV) is appropriate. IGRT improved high-risk target coverage by 1% versus conventional planning (BB) ($p=0.023$). However, mean dose to both parotids increased with IGRT alone. ART1 was able to reduce mean parotid dose relative to IGRT alone in 11/13 cases ($p=0.014$). ART1 significantly reduced the integral body V60Gy and V40Gy by more than 40 cc versus IGRT alone ($p<0.007$). Second replan (ART2) did not reduce parotid or body dose beyond ART1 ($p>0.289$). Thus, we confirm adaptive head and neck radiotherapy to be clinically feasible. IGRT alone does not provide meaningful dosimetric benefit if conventional PTV margins are used. One properly timed adaptive replan (ART1) has provided all relevant dosimetric improvements in this study.

128 Transitioning Basic and Translational Developmental Research of Near-Infrared (NIR) Fluorescence Imaging Into Clinical Trials

Eva M. Sevick-Muraca

Center for Molecular Imaging, The Brown Foundation of Molecular Medicine, The University of Texas Health Science Center, Houston

The ability to non-invasively image the status of disease markers in cancer patients could significantly impact the efficacy of existing and developing therapies. Currently, the “gold-standard” of molecular diagnostic imaging resides with the nuclear medicine imaging. Over the past 15 years, our team has been developing a complementary approach of near-infrared (NIR) fluorescence imaging for the application to cancer nodal staging. NIR fluorescence imaging requires new instrumentation and imaging agents, entailing combinational “first-in-humans” device and drug approvals. Yet before the “risk” of administering a “first-in-humans” imaging agent can be accepted, the “benefit” that arises from its detection deep from within tissues had to be demonstrated with our prototype imaging instrumentation (device). In a completed Phase I dose escalation trial, we employed Indocyanine Green (ICG) in an off-label indication as a non-specific imaging agent to image tumor draining lymph nodes in breast cancer patients. ICG has an established safety record in humans at approved doses of 25 mg IV but is a poor NIR fluorophore. Nonetheless, our prototype device was able to detect fluorescent nodes (estimated as deep as 4–5 cm) and lymphatic vessels at a minimum dose of 10 mcg ID, suggesting the “benefit” of detecting 30 times brighter “first-in-humans” NIR fluorescent imaging agents after microdose administration. In addition, we found that given the device’s high sensitivity, the propulsive lymphatic function and vessel architecture could be visualized for the first time. This ancillary finding has since evolved into two ongoing Phase I/II studies to use NIR fluorescence imaging to assess lymphatic function and architecture in cancer survivors who encounter lymphedema as a result of resection of lymph nodes for diagnostic staging/therapeutic purposes and nodal staging of cancer. Currently “first-in-humans” Phase I trials that employ microdosage of peptide and antibody labeled with IRDye800, a bright, stable NIR fluorophore, for nodal staging of cancer are being developed. By dual labeling with both a nuclear and optical reporter, we seek to develop our technology with a “built in” comparative assessment against conventional, nuclear image modalities.

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129 Standardized Network Communication for MRI-Guided Robotic Prostate Interventions

Clare M. Tempany[1], Gabor Fichtinger[2], E. Clif Burdette[3]

[1]Brigham and Women's Hospital, Boston, MA; [2]Queen's University, Kingston, ON, Canada; [3]AcousticMed Systems Inc., Champaign, IL

An MRI-compatible needle placement robot is an enabling tool for MRI-guided prostate cancer treatment in a high-field scanner. Communication among the robot, MRI scanner, and navigation software, typically through a network, is inevitable for clinical applications, where the robot places the needle as planned on pre-operative images and the process is monitored through real-time MR images. However, there have been few standardized communication protocols designed for MRI-guided robotic interventions. Lack of standard not only forces developers to spend considerable effort on system integration, but it also inhibits replacing prototype components with commercial products, which is essential in translational research. To address this issue, we developed an open, simple, and extensible network communication protocol for a wide range of image-guided therapies (IGTs) named OpenIGTLink. The protocol allows transferring any kinds of data for IGT (e.g., positions, images, and device status) over the TCP/IP network. Based on this protocol, we integrated our MRI-compatible pneumatic needle placement manipulator with a 3T MRI scanner (GE Excite HD 3T, GE Healthcare, Waukesha, WI) and open-source navigation software, 3D Slicer, or commercial software, RadVision (AcousticMed Systems, Champaign, IL). The positions of the target lesion are specified on the navigation software and transferred to the robot control unit. While the robot is maneuvering the needle, its position is calculated from the optical encoders and sent back to the navigation software. The navigation software calculates the imaging plane that intersects the needle's axis and transfers it to the scanner, which in turn acquires semi-real-time images in that plane. Our mockup procedure demonstrated that both navigation software successfully communicated with the robot and the MR scanner with sufficient performance in terms of latency and frequency. We could use 3D Slicer for prototyping the system while we develop RadVision, which has been integrated with intraoperative dosimetry calculation and used in clinical prostate brachytherapy. Therefore, the protocol proved to be useful for transitioning from research prototype to commercial system. Indeed, OpenIGTLink have started being used for several other IGT projects, including neurosurgical and cardiac navigations.

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130 Deformable Image Registration for Accurate Dose Accounting in Liver Radiotherapy

Michael Velec[1], Joanne L. Moseley[1], Laura A. Dawson[1,2], Kristy K. Brock[1,2,3]

[1]Radiation Medicine Program, Princess Margaret Hospital, University Health Network, Toronto, ON, Canada; Departments of [2]Radiation Oncology and [3]Medical Biophysics, University of Toronto, Toronto, ON, Canada

Stereotactic body radiotherapy (SBRT) requires a high degree of precision to treat liver cancer tumors and avoid toxicity to nearby healthy organs. Advances in image-guided SBRT such as cone-beam CT (CBCT) allow internal anatomy to be visualized in three dimensions at the time of treatment. As SBRT delivers greater doses in fewer fractions, the cost of unresolved geometric uncertainties rises relative to conventional treatment. These include respiratory motion, organ deformation, and residual setup inaccuracies that can all vary daily and cause changes to the delivered dose. Deformable image registration of CBCT can be used to accumulated dose, improving understanding in the impact of these uncertainties. Previous patients treated with 6-fraction SBRT were analyzed. Breathing phases extracted from CBCT images provided a breathing model of the patient at each treatment. MORFEUS, a multi-organ biomechanical model-based deformable registration algorithm, was used to deform a three-dimensional model of the patient, created from structures delineated on the planning four-dimensional CT, into each exhale and inhale CBCT. This facilitated dose accumulation by tracking tissue throughout the treatment course. Deviations in tumor and normal tissue doses were quantified. Components of dose accumulation such as variability in breathing motion, deformation, and residual error were analyzed to determine where large sources of uncertainty may exist. Relative to the initial planned dose, accumulated dose changes of at least 1 Gy were seen to tumors for 35% of patients, with a maximum decrease of 5 Gy. Similar changes to normal tissues were seen in 65% of cases up to a maximum change of 21 Gy. Attempting dose accumulation using rigid registration alone (excluding MORFEUS) would have produced errors greater than 1 Gy to 29% of cases for tumors and 71% for normal tissues, up to a maximum of 4 Gy for either. Variability in breathing motion relative to four-dimensional CT caused discrepancies greater than 1 Gy in 47%, up to 4 Gy to tumors and 10 Gy to normal tissue, had the daily breathing motion observed on CBCT not been accounted for in the dose accumulation. Using deformable image registration to accumulate dose over a large patient sample will increase the knowledge of where uncertainties exist in treatment delivery and lead to improvements in treatment planning and daily image-guidance. Reducing uncertainties in delivered dose for SBRT liver will also improve the ability to correlate clinical outcomes in SBRT liver cancer trials that utilize radiation, either alone or in combination with novel agents being researched.

131 A Phase I Trial of ALA PDT for Treatment of Oral Leukoplakia

Stuart J. Wong[1], Bruce Campbell[1], Becky Massey[1], Denis Lynch[2], Ezra E. W. Cohen[3], Borko D. Jovanovic[4], Raymond C. Bergan[4]

[1]Medical College of Wisconsin; [2]Marquette University School of Dentistry; [3]University of Chicago Cancer Research Center; [4]Robert H. Lurie Comprehensive Cancer Center of Northwestern University

Leukoplakia is a premalignant condition involving the oral mucosa that is characterized by a white patch or plaque and coexists with varying grades of dysplasia. Oral leukoplakia is an excellent clinical model for examining cancer prevention strategies. Photodynamic therapy (PDT) involves the topical or systemic administration of a photosensitizing agent that, in the presence of light corresponding to an optimal wavelength, creates reactive oxygen species capable of inducing cytotoxic damage. Aminolevulinic acid (ALA) is an endogenous metabolite that when exogenously administered bypasses the negative feedback control of its metabolic pathway and leads to preferential accumulation of the photosensitizer protoporphyrin IX in mucosal tissues rather than the underlying stroma. We hypothesize that use of ALA PDT for oral leukoplakia is safe and tolerable and that quantitative histologic assessment of response using specific biologic markers (DNA ploidy, proliferation using Ki-67, apoptosis using TUNEL, cyclin D1, p53) is feasible. To test this hypothesis we initiated a Phase I clinical trial to determine the toxicity and feasibility of ALA PDT, assess treatment efficacy by examining clinical and quantitative histologic response, and explore the association of response with specific molecular and biologic markers. The study examines six cohorts in which escalating doses of long pulse dye laser from 2 to 12 J/cm2 (585 nm light, Photogenica SV pulsed dye laser system) are administered with a fixed dose of oral ALA (30 mg/kg). The ALA dose was reduced from 60 mg to 30 mg after a subject in cohort 1 experienced a grade 3 event due to transient liver function test elevation. Enrollment into this trial is ongoing.

132 Exploring the Physiology of Cancer-Related Fatigue

Debra Barton[1], Charles Loprinzi[1], Julienne Bower[2], Elizabeth Breen[2]
[1]Mayo Clinic, Rochester MN; [2]University of California, Los Angeles, CA

Fatigue is the most prevalent symptom experienced by cancer survivors, with up to 90% experiencing fatigue that interferes with usual activity at some point after diagnosis. Fatigue can impact a broad group of patients, irrespective of type of treatment, treatment status, or type of cancer. Evidence-based interventions for fatigue are lacking. One of the barriers to furthering intervention research is an incomplete understanding of the physiology of cancer-related fatigue. Several small studies exist, primarily in breast cancer, that propose dysregulation of both adrenal and inflammatory processes as underlying pathology for cancer-related fatigue. Research has been published that indicates lower cortisol levels, blunted diurnal cortisol rhythms, and decreased cortisol responses to laboratory-induced stressors as well as increases in pro-inflammatory cytokines such as IL-1ra, sTNFRII, and neopterin in women with a history of breast cancer who are fatigued versus those who are not fatigued. Similar physiologic profiles have been identified in other chronic illnesses that have fatigue as a primary component, namely, rheumatoid arthritis, chronic fatigue syndrome, and myelofibrosis. The North Central Cancer Treatment Group has been engaged in intervention research to evaluate pharmacologic agents to reduce fatigue in cancer survivors. Most recently, a placebo-controlled, phase II study demonstrated improvement in fatigue of a half standard deviation effect size in those taking 2,000 mg of Wisconsin ginseng daily versus a placebo. This intervention is being studied in a multi-site phase III trial in survivors with many types of cancer who are being or have been treated with curative intent. The active trial includes a translational component whose primary aim is to describe the cortisol and cytokine values in fatigued survivors with various types of cancer. Further, it will evaluate whether Wisconsin ginseng impacts the expression of cortisol and cytokines in this population. Participants who are not receiving antineoplastic cancer treatment are providing saliva and blood at baseline, before starting ginseng or placebo, and at 4 weeks after starting the study agent. Our working hypotheses are that fatigue scores, levels of pro-inflammatory cytokines, and flattened cortisol slopes will be highly correlated at baseline and that improvements in fatigue will correlate with lower levels of cytokines and improved cortisol slopes and be attributable to treatment with Wisconsin ginseng.

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133 Take Heart: Exercise Intervention for Heart Failure in Cancer Survivors

Karen Basen-Engquist, Daniel C. Hughes, Jennifer Jovanovic, Carol Harrison, Daniel J. Lenihan
The University of Texas M. D. Anderson Cancer Center

Background: Cardiac toxicity is a troubling effect of cancer treatment, affecting quality of life of survivors and limiting use of life-saving therapy. Exercise interventions have been helpful in heart failure patients as well as cancer survivors without heart failure. However, lifestyle interventions have not been tested in cancer survivors with heart failure due to chemotherapy-induced cardiac toxicity.

Purpose: Our long-term goal is to conduct a randomized control trial of an exercise and diet intervention for cancer survivors with chemotherapy-related heart failure. Here we present the results from our first two pilot study participants.

Methods: We conducted a 16-week program involving exercise and sodium intake counseling, assessing fitness, symptoms, and heart failure progression at baseline and post-intervention. To develop the exercise prescription, we used the heart rate reserve (HRR) method coupled with rating of perceived exertion (Borg RPE) with the goal to increase exercise duration to 30 minutes of continuous exercise at a level of at least 50% HRR.

Results: Two participants have completed the initial feasibility test: (1) a 56-year-old female survivor of Hodgkin's lymphoma and (2) a 46-year-old male survivor of leukemia. Both developed heart failure as a result of chemotherapy. Both patients experienced improvements in exercise tolerance. Participant 1 improved from 11.5 minutes of exercise split over two bouts at an RPE of 14 in the first week to 30 minutes in one bout at an RPE of 15. Participant 2 improved from 11 minutes in two bouts to 18 minutes in one bout (RPE 12). Both participants had improvements in VO2 peak (Patient 1: 13.9 to 14.3 mlO2/kg/min; Patient 2: 12.5 to 18.7 mlO2/kg/min) with ventilatory threshold improving from 80% to 95 % of VO2 peak for Patient 1 and decreasing from 78% to 66% of VO2 peak for Patient 2. Symptoms rated on a 0–10 scale decreased from baseline to 4 months (Patient 1: 3.3 to 2.2; Patient 2: 5 to 3.3; 0="no symptoms", 10="as bad as can be imagined"). Ejection fraction did not change for Patient 1 (35–40%) but increased for Patient 2 (25–30% to 35–40%). BNP improved for Patient 1 (124 to 56 pg/ml) but not Patient 2.

Conclusions: To our knowledge, there have been no published reports of cardiac rehabilitation specifically for cancer survivors with heart failure. These initial case studies indicate that this is an important area for future research to improve functioning and quality of life for this population of survivors.

134 Transgenic Supermouse Behavior Model of Enhanced Exercise to Determine Intermediary Markers for Cancer Prevention Targets

Nathan A. Berger, Richard W. Hanson, Joseph H. Nadeau, Parvin Hakimi, Gemma Casadesus, James H. Swain

Case Center for Transdisciplinary Research in Energy Balance and Cancer, Case Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, OH

Our overall goal is to exploit the unique phenotypic characteristics of the phosphoenol pyruvate carboxy kinase C (PEPCK-Cmus) transgenic mouse as an animal model to investigate the impact of enhanced physical activity on the development and progression of gastrointestinal neoplasia and to identify target intermediary markers for translating this behavioral modification to humans. The PEPCK-Cmus TG mouse contains the gene for the cytoplasmic form of the phosphoenolpyruvate carboxy kinase (PEPCK-Cmus) driven by the α skeletal muscle actin gene promoter, which generates an animal that markedly overexpresses PEPCK-C in skeletal muscle. These mice are 10 times more active in their home cages than control animals and can run up to 6 km at a speed of 20 m/min on a mouse treadmill without stopping. This enhanced exercise phenotype is apparent during the entire lifespan of the transgenic mice. PEPCK-Cmus mice eat twice as much as controls but weigh less; they live longer, have stronger bones, demonstrate sustained fertility, and show less spontaneous malignancies. Studies of the enhanced exercise phenotype on intestinal tumor development progression and survival induced by the APCmin mutation show that the behavioral alteration is associated with delayed tumor progression and prolonged survival in both males and females. Examination of metabolic, cytokine, adipokine, hormonal, and hematologic parameters reveal unique patterns associated with the enhanced exercise phenotype. These patterns should serve as the basis for translational studies to evaluate and set targets for intermediary markers in trials of exercise and other biobehavioral interventions for cancer prevention and control in humans.

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135 A Translational Pathway for Reducing the Symptoms and Toxicities of Cancer Therapy

Charles S. Cleeland

Department of Symptom Research, The University of Texas M. D. Anderson Cancer Center

Despite progress in developing new approaches to cancer therapy, many patients (as many as 40% in a recent study) have to delay treatment schedules or terminate therapy due to symptoms (especially fatigue, joint pain, and neuropathy) and toxicities (cardiac, pulmonary, and dermatological) of therapies. Few studies on the impact of dose reduction or treatment terminations due to the symptoms or toxicities of treatment on time-to-disease-progression or on overall survival have been conducted, but the potential impact on both of these outcomes is liable to be large. In addition, the negative impact of persistent treatment-related symptoms and toxicities on cancer survivors on their resumption of social and vocational function is becoming evident. At this point, there are few programs with a primary focus on treatment-related symptoms and toxicities. A translational pathway having the same key features as pathways for curative and diagnostic agents could guide the development of therapies to reduce or prevent the unwanted effects of treatment. Animal models for neuropathy associated with chemotherapy have already been developed. There is a need to develop and test other animal models of cancer-related symptoms (fatigue, sleep disturbance) and toxicities (cardiovascular, pulmonary, and neurological). Several disciplines are well poised to contribute to this pathway. There is now substantial evidence that genetics play a role in the severity of symptoms toxicities that result from therapy, as well as in predicting the relative success of symptom treatment. Proteins associated with symptom severity have been identified. Molecular imaging could also be deployed to investigate mechanisms underlying these symptoms/toxicities caused by treatment. Finally, patient reports such as symptom ratings are now well accepted as outcomes in early phase clinical research. There is a need to create a multidisciplinary translational working group focused on the reduction of the symptoms and toxicities of cancer therapy. This group, including cancer patients and survivors, would identify and prioritize critical steps (molecular studies, animal models, and pre-clinical studies in humans) and candidate therapies for phase I/II clinical trials, with the goal of increasing the tolerability of cancer treatment and preventing or greatly reducing treatment-related long-term symptom burden and medically significant late effects.

136 RENEW: Reach-Out to ENhancE Wellness in Older Cancer Survivors

Wendy Demark-Wahnefried[1], Miriam Morey[2], Denise Snyder[2], Richard Sloane[2], Bercedis Peterson[2], Harvey J. Cohen[2]
[1]The University of Texas M. D. Anderson Cancer Center, Houston, TX; [2]Duke University Medical Center, Durham, NC

Currently, there are over 12 million U.S. cancer survivors. Given advances in early detection and treatment, this number is expected to increase by roughly 300,000 each year. More than 60% of these survivors are at least 65 years old (since cancer is a disease associated with aging), and most are survivors of breast, prostate, and colorectal cancer (since the 5-year "cure" rates for these cancers now exceeds 90% for early-stage disease). The increase in the number of cancer survivors is good news, but the impact of cancer is significant and associated with several adverse long-term health and psychosocial sequelae. Compared to others, cancer survivors are at significantly greater risk for second cancers, cardiovascular disease, osteoporosis, diabetes, and functional decline. For older survivors, whose functional status may already be marginal, further declines in function can threaten independence and incur significant burden and health care costs. The purpose of the RENEW trial was to determine if a year-long, diet-exercise intervention delivered to older, overweight, long-term cancer survivors via telephone prompts, counseling, and mailed materials could improve health behaviors and result in improved weight status and physical function (PF). Rates of functional decline (as measured by the SF-36 physical function subscale and the Late-Life basic and advanced scales) were significantly reduced in the intervention arm, as compared to the wait list control, i.e., -2.55 (1.07) versus -5.39 (1.01), p=0.034; +0.41(0.71) versus -2.11(0.67), p=0.005; and +0.44 (0.60) versus -2.55 (0.61), p=0.14, respectively. Furthermore, the intervention resulted in significant improvements in diet quality, and strength and endurance exercise. Weight loss also was significantly greater in intervention as compared to the control arm, -2.5 (3.5) versus -1.0 (3.9) kg, respectively (p<.0001). Thus, this intervention was significantly effective at improving lifestyle behaviors and health-related outcomes among older cancer survivors. Future work is slated to assess the durability of the intervention, explore the potential for further dissemination, and perform cost-benefit analyses. We also hope to adapt the intervention to other modalities (e.g., web-based, smart-phone) and determine impacts not only in this population but also in other survivorship and high-risk populations.

137 Biological and Behavioral Responses to a Mindfulness-Based Intervention in Women at Increased Risk for Cervical Cancer

Carolyn Y. Fang[1], Diane K. Reibel[2], Norman G. Rosenblum[2], Mark A. Morgan[1], Cynthia Bergman[1], Stephen Rubin[3], Karthik Devarajan[1], Donald E. Campbell[4], Steven D. Douglas[4]
[1]Fox Chase Cancer Center; [2]Thomas Jefferson University Hospital; [3]University of Pennsylvania Health System; [4]The Children's Hospital of Philadelphia

Mindfulness-based stress reduction (MBSR) is a standardized program that has been demonstrated to have beneficial effects on health outcomes in diverse medical populations. The core of MBSR is intensive training in mindfulness meditation and its applications for daily living and coping with stress, pain, or illness. Studies have consistently demonstrated that MBSR not only leads to improvements in quality of life and reductions in distress, anxiety, and medical symptoms, but also is associated with significant alterations in various immune parameters, including enhanced cell-mediated immunity and decreased levels of pro-inflammatory cytokines. These findings are relevant in light of empirical evidence that immune factors play a central role in controlling human papillomavirus (HPV) infection and cervical disease progression. Thus, we are currently conducting a randomized clinical trial to evaluate the effects of a standardized 8-week MBSR program, compared to a control condition, on HPV-specific immune functioning among women with cervical dysplasia. To assess immune response to HPV16, synthetic HPV16 peptide sequences spanning the entire length of the HPV16 E6 and E7 proteins are combined into seven peptide pools and used as antigens in lymphoproliferative assays, which are conducted using fresh blood samples. Specific peptide sequences of particular interest have also been maintained as separate peptide antigens. In addition, we are using the flow cytometry-based cytometric bead assay (CBA) to evaluate HPV peptide-induced Th-1/Th-2 cytokine production in supernatants of cultured peripheral blood mononuclear cells (PBMCs) obtained from study participants at pre- and post-program assessments. The cytokines to be evaluated include INF-gamma, TNF-alpha, IL-2, IL-10, IL-5, and IL-4. Optimal cytokine production was observed in the evaluation of culture fluids from antigen-stimulated PBMCs harvested at 48 hours. Cytokine profiles will be correlated with subsequent lymphoproliferative responses as evaluated by HTdR uptake. Interim analyses are ongoing and will also examine women's psychosocial and behavioral responses following participation in MBSR. These data will contribute to a greater understanding of how alterations in biobehavioral pathways may influence susceptibility to cervical cancer in an at-risk population.

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138 Patient Drop-Out From Cancer Treatment: Comparison of Rural and Urban Underserved Areas

Theresa W. Gillespie, Joseph Lipscomb, Kevin Ward, Michael Goodman

Emory University Winship Cancer Institute

Background: Recent patterns-of-care studies report that cancer patients often do not complete prescribed therapy, with potentially important implications for clinical outcomes including survival. Yet, little is known about the frequency and determinants of early treatment discontinuation. The aims of this study were to address these issues in a largely rural population of a 33-county region of southwest Georgia.

Methods: A total of 3,996 cases of breast, colorectal (CRC), lung, and prostate cancer diagnosed between 2001 and 2003 were identified from the Georgia Comprehensive Cancer Registry, with data collected from medical records by trained abstractors; 3,949 cases were available for analysis. Since completion of adjuvant chemotherapy with a goal of cure may be particularly important, this report focuses on cases treated for stage I and II breast cancer and stage III CRC.

Results: Among 854 stage I–II breast cancer cases, 323 (38%) received chemotherapy. In 293 of those cases, treatment was stopped within the first year post-diagnosis. The primary reasons for stopping included treatment completion (86%), significant toxicity (8%), or other clinical reasons (2%). In only seven cases (2%) was treatment discontinued due to patient factors (e.g., refusal or did not return to clinic). Among 179 stage III CRC cases, 124 (69%) received adjuvant chemotherapy; in 117 cases, chemotherapy was stopped within 12 months following diagnosis. Clinical reasons for stopping chemotherapy in those 117 cases included treatment completion (67%), toxicity (12%), disease progression (8%), or death (3%). In only 4% of cases was chemotherapy discontinued due to patient refusal, and in 3% of cases a patient was lost to followup. Rural results were then compared with an urban underserved population in Atlanta, Georgia.

Conclusions: In this largely rural population, only a small proportion of cases stopped adjuvant chemotherapy early due to patient-attributable factors; most treatment termination was due to clinical indications. Review of practice patterns using only administrative data may not reveal the actual reasons for treatment discontinuation. Further study of underlying factors of early termination is needed to design appropriate interventions to promote completion of prescribed therapy.

139 Walking Forward Program: Participation of American Indians in Cancer Clinical Trials

Shalini Kanekar, Daniel Petereit

Cancer Care Institute, Rapid City Regional Hospital, Rapid City, SD

Walking Forward is a community-based participatory research program in western South Dakota funded by the National Cancer Institute. The primary goal of this initiative is to address the high and ominously increasing cancer mortality rates among American Indians by facilitating access to innovative clinical trials, behavioral and genetic research, and tailored patient navigation. The critical outcomes include: (a) a high accrual rate in clinical trials, including cancer treatment and cancer control trials; (b) a significant reduction in the number of missed treatment days among navigated American-Indian cancer patients undergoing radiation therapy; and (c) most importantly, establishment of trusting partnerships with the American-Indian communities as reflected in enrollment in a genetic study involving the ataxia telangiectasia mutated gene. The results indicate that the Walking Forward approach presents an effective strategy to overcome the barriers to cancer care in this underserved community.

140 Risk of Second Cancers Following Treatment for Retinoblastoma Since 1970

Ruth A. Kleinerman[1], Ritsu Sakata[1], David H. Abramson[2], Johanna Seddon[3], Peggy Tucker[1]

[1]National Cancer Institute; [2]Memorial Sloan-Kettering Cancer Center, New York, NY; [3]Tufts Medical Center, Boston, MA

Children with hereditary retinoblastoma (RB) (i.e., those with a germline mutation in the RB1 tumor suppressor gene) survive into adulthood but are at increased risk of developing second primary cancers due to genetic predisposition, which is enhanced by radiotherapy. To evaluate whether children with retinoblastoma treated since 1970, when radiotherapy doses decreased and chemotherapy use increased, are at risk of developing second cancers, we analyzed incident cancer risk in a large cohort of 867 RB survivors treated 1970–96 at two institutions. The observed number of second cancers was compared to the expected based on general population rates (standardized incidence ratio [SIR]). We also estimated the relative risk (RR) of second cancers associated with sex, age at retinoblastoma, attained age, hereditary status, and treatment. Since 1970, 56 second cancers were diagnosed in 476 hereditary (SIR=30, 95%, CI 23–39) and two cancers in 391 non-hereditary patients. The highest risks occurred 10–19 years after retinoblastoma (SIR=51, 95%, CI 34–74), mainly for cancers arising in the radiation field, including bone and soft tissue sarcomas of the head. Risks for second cancers were associated with hereditary versus non-hereditary disease (RR=18, p=0.001); any radiation versus no radiation (RR=12, p=0.001); and any chemotherapy versus no chemotherapy (RR=1.7, p=0.07). We noted a 40% lower risk of second cancers in hereditary patients irradiated when over 12 months of age. Patients treated after 1970 experienced a nonsignificant 20% lower relative risk of second cancers compared with those treated in earlier decades. Hereditary status and radiotherapy remain major determinants of second cancers. Although retinoblastoma patients treated in the past few decades appear to have a lower risk of second cancers than children treated in an earlier time period, second cancers still persist in this population.

141 Depression, Loneliness, Angiogenesis, and Invasion in Ovarian Carcinoma

Susan K. Lutgendorf[1], Lauren Clevenger[1], Koen DeGeest[1], Frank Penedo[2], Joseph A. Lucci, III[3], Steve W. Cole[4], Anna Lokshin[5],David M. Lubaroff[1], Anil K. Sood[6]

[1]University of Iowa; [2]University of Miami; [3]University of Miami Sylvester Cancer Center; [4]University of California, Los Angeles; [5]University of Pittsburgh Cancer Center; [6]The University of Texas M. D. Anderson Cancer Center

Emerging data has highlighted relationships between biobehavioral factors and molecules involved in angiogenesis and invasion in ovarian cancer. Vascular endothelial growth factor (VEGF), a key promoter of tumor angiogenesis, is associated with poor ovarian cancer survival and is known to be stimulated by the stress hormones norepinephrine (NE) and cortisol. Anti-angiogenic factors such as angiostatin and endostatin are part of a delicate balance controlling angiogenesis. Matrix metalloproteinases (MMPs) -2 and -9 are critical to ovarian cancer invasion, and their production by ovarian tumor cells is also stimulated by NE. Patients having surgery for suspected ovarian cancer were recruited to this study and completed questionnaires within a week prior to surgery. Ninety-three patients were included following histological confirmation of epithelial ovarian cancer and assessment of tumor NE. Tumor samples (n=56) were analyzed for tumor-associated macrophage (CD68+) and tumor cell production of MMPs-2, -9 and VEGF using confocal microscopy and were assessed for tumor NE using HPLC (n=93). Plasma samples (n=20) were examined for anti-angiogenic molecules (endostatin, angiostatin) using luminex. All analyses adjusted for cancer stage. Patients with higher levels of depressed mood and lower social support had elevated in-tumor NE ($p<0.05$). Depressed mood was also associated with lower levels of plasma angiotensin ($p<.05$), and greater life stress was associated with lower endostatin ($p<.01$). Greater depressed mood ($p<0.0001$) and life stress over the last 6 months ($p=0.004$) was related to greater MMP-9 in tumor-associated macrophages (CD68+), and loneliness was associated with greater tumor production of MMP-9 ($p=0.023$) and VEGF ($p=0.036$). These data demonstrate relationships between stress, depression, and loneliness with adrenergic factors in tumors, with lower availability of anti-angiogenic factors in plasma, and with higher levels of pro-angiogenic and invasive factors at the level of the tumor. These findings coupled with our experimental findings suggest that biobehavioral factors may directly influence angiogenesis and invasion and thus may influence tumor progression in ovarian cancer. Findings have implications for intervention strategies for ovarian cancer patients.

142 Modifiable Lifestyle Interventions in Patients at Higher Risk for Young-Onset Pancreatic Cancer

Robert McWilliams, William Bamlet, Kari Rabe, Martha Matsumoto, Mariza de Andrade, Edward Highsmith, Gloria Petersen

Mayo Clinic

Background: Modifiable lifestyle factors including body mass index (BMI) and smoking have been associated with risk for pancreatic cancer, but effects of age of onset had not previously been established. Patients at genetic risk for pancreatic cancer can be identified, but the impact of lifestyle modifications for these patients had been unclear. This project has relevance to both the "Lifestyle Alterations" and "Biospecimen-Based Assessment Modalities" pathways.
Methodology: We have utilized the Mayo Clinic Pancreas Biospecimen Resource, funded by SPORE grant P50 CA 102701, to examine the effect of these modifiable lifestyle factors and genetic risk factors on age of onset of pancreatic adenocarcinoma. Participants complete risk factor questionnaires and have biospecimens collected, including lymphocyte DNA. **Results:** Modifiable lifestyle factors that we have associated with an earlier age of pancreatic cancer diagnosis include elevated BMI and smoking. Both have exhibited a dose-dependent effect on earlier ages at diagnosis. In addition, at least one genetic risk factor (being a carrier/heterozygote for a mutation in the cystic fibrosis transmembrane regulator gene [CFTR]) has also shown an association with younger onset disease, exclusively among smokers. **Conclusions:** Our work on younger-onset pancreatic cancer has public health implications for lifestyle alterations combined with genetic biomarkers. It is clear that risk for cancer alone does not have adequate impact for modification of risk at the population level, but the identification of modifiable risk factors has real potential for prevention, particularly when focused on motivated high-risk groups such as family members of pancreatic cancer patients. This may also include people at increased genetic risk for pancreatic cancer, such as those with a family history of pancreatic cancer. We have demonstrated that CFTR mutations are associated with a younger onset of disease only in smokers, so tobacco cessation is a direct intervention that can be recommended for identified carriers of mutations concerned about their risk. In addition, maintenance of a healthy weight also has potential to decrease risk for younger-onset pancreatic cancer. Combination of modifiable lifestyle risk factors and identification of those at increased risk via genetic biomarkers can be combined to provide useful tools for clinicians. Patients at genetic risk can be identified, and concrete recommendations on maintaining a healthy weight and not or stopping smoking can be made to decrease their personal risk for pancreatic cancer.

143 TMPRSS2:ERG and SPINK1 in Prostate Cancer Etiology and Progression

Lorelei Mucci[1,2,3], Richard Flavin[1,4], Azra Ligon[2,4], Kathryn Penney[1,2,3], Whitney Hendrickson[1,4], Michelangelo Fiorentino[4], Jennifer Stark[1,2,3], Neil Martin[2,4], Blaine Philips[2], Christopher Fiore[4], Jennifer Sinnott[2,3], Jing Ma[1,2], Matthew Freedman[1,4], Stephen Finn[4], Edward Giovannucci[1,2,3], Meir Stampfer[1,2,3], Massimo Loda[1,4], Philip Kantoff[1,2,4]

[1]Dana-Farber Cancer Institute/Harvard Cancer Center Prostate Cancer Program; [2]Brigham and Women's Hospital and Harvard Medical School; [3]Harvard School of Public Health; [4]Dana-Farber Cancer Institute, Boston, MA

ERG and SPINK1 were nominated as top oncogenes in prostate cancer (PCa) through a Cancer Outlier Profile Analysis, an approach that led to discovery of the TMPRSS2:ERG fusion. SPINK1 outlier expression appears as a distinct molecular subset in tumors that lack TMPRSS2:ERG. Some studies suggest both TMPRSS2:ERG- and SPINK1-positive tumors have a more aggressive phenotype. However, not all men who harbor the molecular alterations progress, while some with neither develop disseminated disease, suggesting additional factors are necessary. We are undertaking a comprehensive patho-epidemiology study to explore germline variants, lifestyle factors, or additional markers with TMPRSS2:ERG and SPINK1 in PCa risk and progression. The study is nested among 1,500 men with PCa (1983–2004) in the Physicians' Health Study and Health Professionals Follow-Up Study. The men were followed for bony metastases and cancer-specific mortality through 2008; 175 have developed lethal disease. We are characterizing TMPRSS2:ERG and SPINK1 on archival tumor tissue specimens. Germline SNPs in genes involved in sex hormone metabolism and IGF/insulin pathways were assayed. Lifestyle information was collected pre- and post-diagnosis. Expression of additional markers was assayed by IHC. The TMPRSS2:ERG prevalence was 42%, 2/3 by deletion. Twelve percent of tumors were SPINK1-positive; 1% shared both alterations. The fusion prevalence did not differ by Gleason grade. However, tumors diagnosed at an advanced (64%) versus localized (39%) stage were more likely to be fusion-positive. Fusion-positive tumors, particularly by deletion, had substantial reductions in tumor apoptosis ($p=0.03$). Neither fusion or SPINk1 status predicted lethal outcomes. Fusion-positive tumors showed increased sex hormone signaling, with upregulation of AR ($p\sim0.02$) and ER-alpha ($p<0.001$). On a subset of tumors (n=116) with RNA expression data, an 87-gene signature differentially expressed in fusion-positive and negative tumors was identified to be related to estrogen signaling. TMPRSS2:ERG tumors may also respond to insulin signaling. Fusion-positive tumors had considerable upregulation of tumor expression of insulin receptor and IGF1R, a finding intriguing since obese men had a lower risk of developing fusion-positive tumors compared to men at a healthy weight (RR=0.32, 95% CI=0.12-0.81). Future work will focus on understanding the role of sex hormones and insulin on prostate cancer progression in combination with the fusion.

144 Energy Balance, Leptin, and Prostate Cancer Risk in the Prostate Cancer Prevention Trial

Marian L. Neuhouser[1], Cathee Till[1], Phyllis Goodman[1], Alan R. Kristal[1] and Michael Pollak[2]

[1]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle WA; [2]Departments of Medicine and Oncology, McGill University, Montreal, Canada

Overweight and obese men are at increased risk for high-grade prostate cancer. In addition, overweight and obese men have poor prognosis and die of prostate cancer more often than normal-weight men. Understanding the biology of energy imbalance in relation to both risk and prognosis is an important translational science topic with bench-to-bedside applications. For example, lifestyle modifications or effective pharmaceuticals may be part of the treatment portfolio, but in order to devise the best therapies, a better understanding of the obesity-carcinogenesis biology is needed. To this end, we have examined the role played by leptin in relation to prostate cancer and the extent to which leptin may help explain obesity and cancer relationships. Leptin is synthesized by adipose cells, as well as other tissues; leptin levels tend to be higher in overweight people due to their overabundance of adipose cells. Leptin has numerous functions including regulation of energy intake via its effect on satiety, modification of insulin sensitivity, and regulation of the immune response and inflammation. Here we examined the association of baseline measures of serum leptin with risk of total, low-grade, and high-grade prostate cancer in the Prostate Cancer Prevention Trial (PCPT). Multivariate-adjusted logistic regression tested associations of BMI and serum leptin with total prostate cancer risk and multivariate-adjusted polytomous regression modeled low-grade (Gleason<7) and high-grade disease (Gleason>7). We observed inverse associations of obesity with low-grade prostate cancer (OR=0.78, 95% CI, 0.62–0.96, p, trend=0.02). However, men who were obese (BMI>30.0) had an increased risk of high-grade prostate cancer (Odds Ratio=1.36, 95% CI, 1.01–1.82, p, trend=0.04). High versus low serum leptin was associated with a 25% reduced risk of total prostate cancer (p, trend=0.01), and risk estimates were similar for low- and high-grade disease. We next examined whether leptin mediated the obesity-cancer associations, even though leptin alone did not appear to be a risk factor. We observed no evidence that leptin mediated the association of obesity with high-grade prostate cancer. We conclude that obesity is a strong risk factor for high-grade prostate cancer in the PCPT, but the biological mechanism is not via leptin. Clinicians should advise men to maintain a healthy weight to reduce prostate cancer risk; further work is needed to understand the biological mechanisms.

145 Rapid Genetic Counseling and Testing for Newly Diagnosed Breast Cancer Patients

Marc D. Schwartz[1], Heiddis Valdimarsdottir[2], Beth Peshkin[1], Claudine Isaacs[1], Shawna Willey[1], Lina Jandorf[2]

[1]Lombardi Comprehensive Cancer Center; [2]Mount Sinai School of Medicine, New York

Breast cancer patients who carry a BRCA1 or BRCA2 mutation have up to a 65% lifetime risk of developing a contralateral breast cancer, and as a result, bilateral mastectomy (BLM) has emerged as a treatment option for these patients. Although guidelines recommend genetic counseling referral for individuals with a family history suggestive of hereditary cancer, the guidelines do not indicate when patients should be referred relative to their definitive breast cancer treatment. The primary argument for referral prior to definitive breast cancer surgery is that knowing one's BRCA status might influence treatment decisions. Mutation carriers might opt for BLM rather than lumpectomy or unilateral mastectomy. Advantages of immediate BLM may include a reduced rate of contralateral breast cancer, avoidance of radiation treatment, enhanced reconstructive options, avoidance of additional future surgery, and possible cost savings from duplicative treatments. On the other hand, genetic counseling and testing at the time of diagnosis could delay primary breast cancer surgery or cause additional distress during the already stressful post-diagnostic period. Further, most patients will receive uninformative test results that may provide little treatment guidance while potentially leading to treatment delays and added costs. In a non-randomized clinical study, we demonstrated the feasibility of delivering rapid pre-surgical BRCA1/2 genetic counseling and testing. More than one-half of the patients who were found to carry a BRCA1/2 mutation prior to their definitive surgery opted for immediate BLM. Immediate BLM was not associated with adverse psychosocial or quality of life outcomes at 1 year post-diagnosis. In a follow-up randomized controlled trial, we are enrolling all newly diagnosed breast cancer patients with at least a 10% prior probability of carrying a BRCA1/2 mutation. These participants are randomized to receive rapid genetic counseling and testing (RGC) versus usual care (UC). RGC consists of a pre-surgical genetic counseling delivered within 2 weeks of diagnosis, the opportunity to undergo immediate BRCA1/2 gene testing, and rapid disclosure of the test result. UC consists of usual clinical care. Key outcomes include BRCA1/2 testing decision, breast cancer surgery decision, time from diagnosis to definitive surgery, quality of life, and projected cost per quality adjusted life year saved. The results of this trial will provide much-needed clinical data to guide the translation of BRCA1/2 testing into the breast cancer surgery setting.

146 Behavioral Counseling and Varenicline Treatment for Smoking Cessation: Results From a Randomized Trial in a Managed Care Setting

Gary E. Swan[1], Lisa Jack[1], Jennifer B. McClure[2], Sheryl Catz[2], Susan Zbikowski[3], Tim McAfee[3], Mona Deprey[3], Harold Javitz[1], Julie Richards[2]

[1]SRI International; [2]Group Health Center for Health Studies; [3]Free and Clear, Inc.

We will present the results of the COMPASS trial, which compared the effectiveness of three different modes of behavioral counseling, each paired with varenicline. Randomized clinical trials of this medication's efficacy relied on concurrent traditional, one-on-one or group-based supportive counseling. Since this mode of delivery is of limited feasibility for large-scale delivery, it was of interest to determine cessation rates associated with varenicline as coupled with commonly available support through either a proactive telephone-based program, Web-based program, or a program that offered combined telephonic and Web-based intervention. Smokers ready to quit were recruited from Group Health, a large regional health plan with more than 550,000 members. After screening, eligible smokers (n=1202) were randomized to one of three treatment groups (n=400 each), with all participants receiving varenicline by mail (standard 12-week course, ramping up to 2mg/daily following the first week of treatment). Smoking status and other relevant characteristics were assessed at 21 days, 3 months, and 6 months following the target quit date. Results will be described in each of four areas: (a) treatment utilization—utilization of each form of cessation counseling and pharmacotherapy; (b) smoking cessation outcome—7-day point prevalence of smoking outcomes by treatment group and patient-level characteristics predictive of outcome; (c) drug side effects—prevalence and intensity of varenicline side-effects and nicotine withdrawal as a function of participants' lifetime depression history; and (d) cost effectiveness—cost, cost per additional 6-month nonsmoker and per additional lifetime smoker, cost per life-year and per quality-adjusted life-year for each of the three treatment combinations. Additional analyses are underway to identify: (e) interactions between indicators of behavioral and pharmacological treatment utilization and their association with smoking cessation outcome and (f) the relationship between genetic variation and subsequent treatment outcome.

147 An Interactive Colorectal Cancer Screening Promotion Intervention for Latinos

Armando Valdez[1], Marilyn Winkleby[2], Rene Salazar[3], Susan Stewart[4], Maria Fernandez[5]

[1]HealthPoint Institute, Mountain View, CA; [2]Stanford Center for Research and Disease Prevention, Stanford University School of Medicine; [3]Division of General Internal Medicine and [4]Comprehensive Cancer Center, University of California, San Francisco; [5]Center for Health Promotion and Prevention Research, University of Texas, Houston

Colorectal cancer (CRC) is the nation's third most common cancer diagnosed and the second leading cause of death from cancer. An estimated 40% of the total eligible population has not been screened for CRC. The rate of unscreened persons is even greater among the medically underserved, which places them at greater risk for CRC mortality. CRC mortality is a persistent and formidable health disparity experienced by Latinos that has not changed significantly over time due in large part to low screening rates; the result is high incidence of late-stage CRC diagnosis and lower survivorship. This disparity underscores the urgent need for an effective screening promotion intervention for Latinos. Notably, an effective delivery platform has been developed to deliver an individualized, self-paced, bilingual (Spanish/English) cancer screening promotion intervention to low-income, low-literacy Latinos in community clinic settings. Our randomized efficacy studies with breast and cervical cancer education interventions delivered through interactive, multimedia touchscreen kiosks suggest they significantly improve knowledge, attitudes, and screening behavior. Indeed, both studies resulted in adoption of screening behaviors by over 50% of persons exposed to these interventions. These effect sizes suggest that cancer education kiosks have the potential to sufficiently impact Latino cancer screening and early detection rates if diffused nationally at a sufficiently large scale among community clinics that serve predominantly low-income Latinos. These cancer screening promotion kiosks offer a practical and cost-efficient approach to improving Latino CRC screening and reducing CRC mortality. We are currently conducting a randomized controlled efficacy trial to evaluate the potential for CRC screening promotion kiosks to significantly improve Latino screening rates.

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148 Stress, Immunity, and Cervical Cancer: Biobehavioral Outcomes of a Randomized Trial

Lari Wenzel, Edward Nelson, Kathryn Osann, Bradley Monk

University of California, Irvine

A cancer diagnosis and treatment imparts chronic stressors impacting quality of life (QOL) and basic physiology. However, the capacity to increase survival by improving QOL is controversial. Cervical cancer patients, in particular, have severely compromised QOL, providing a population well suited for the evaluation of novel psychosocial interventions and exploration of mechanisms by which modulation of the psychoneuroimmune axis might result in improved clinical outcomes. A randomized clinical trial was conducted in cervical cancer survivors, enrolled at 13–21 months after diagnosis (n=50), comparing a unique psychosocial telephone counseling (PTC) intervention to usual care. QOL and biological specimens (saliva and blood) were collected at baseline and 4 months post enrollment. The PTC intervention yielded significantly improved QOL (p=0.011). Changes in QOL were significantly associated with a shift of immune system T helper type (Th)1:Th2 bias, as measured by Interferon gamma and Interleukin- (IL-) 5 ELISpot T lymphocyte precursor frequency, with improved QOL associated with increased Th1 bias (p=0.012). Serum IL-10 and the neuroendocrine parameters of cortisol and DHEA revealed trends supporting this shift in immunologic stance and suggested a PTC mediated decrease of subject's chronic stress response. This study documents the utility of a unique PTC intervention and an association between changes in QOL and adaptive immunity (T helper class). These data support integration of the chronic stress response into biobehavioral models of cancer survivorship and suggest novel mechanistic hypotheses by which interventions leading to enhanced QOL could result in improved clinical outcome including survival. In support of these significant biobehavioral results, our ongoing follow-up study tests the hypotheses that compared to usual care, cervical cancer survivors (n=250) randomized to PTC will have improved QOL and reduced distress, reduced Th2 immune response, and increased Th1 response. This may have significant implications for clinical endpoints in this underserved population of cancer survivors.

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149 Discovery and Validation of Molecular Targets, Biomarkers, and Non-Toxic Dietary Interventions for Cancer Prevention

Matthew R. Young[1], Gerd Bobe[1,2], Roycelynn Mentor-Marcel[1,2], Terry Hartman[3], Robb Chapkin[4], Elaine Lanza[1,5], John Milner[6], Young Kim[6], **Nancy H. Colburn**[1]

[1]Laboratory of Cancer Prevention, Center for Cancer Research, National Cancer Institute; [2]Preventive Oncology Program, National Cancer Institute; [3]Pennsylvania State University; [4]Texas A&M University; [5]Cytonix; [6]Division of Cancer Prevention, National Cancer Institute

The Gene Regulation Section of the Laboratory of Cancer Prevention (LCP) at the Center for Cancer Research, National Cancer Institute, is focused on understanding the early events in tumorigenesis with the hope of finding new molecular targets, biomarkers, and non-toxic interventions that will prevent or significantly delay cancer. Our work in vitro and with genetically engineered mice (GEM) identified the transcription factor AP-1 as a molecular target for cancer prevention (Young et al., 2003). Mice that express dominant negative c-Jun (TAM67) are protected from environmental and oncogene-induced skin and breast cancer without any noted toxic side effects (Young et al., 2003, Shen et al., 2008). A high-throughput screen has identified novel natural compounds that target AP-1 and/or NFκ B without inhibiting cell proliferation or survival (Ruocco et al., 2007, Kang et al., 2009). The LCP has established multiple collaborations to identify dietary factors that prevent cancer. In collaboration with Michigan State University, Pennsylvania State University, and Texas A&M University, we have been identifying potential molecular targets and biomarkers of efficacious response to dry bean-based diets in parallel in human dietary intervention trials and mouse models of colon carcinogenesis. These studies are based on our observation that high dry bean intake decreased advanced colorectal adenoma recurrence in humans (Lanza et al., 2006) and protected against chemically induced colon carcinogenesis in obese mice (Bobe et al., 2008). So far, IL-6 has been identified as a potential biomarker and molecular target. Serum IL-6 concentrations were elevated in participants of the Polyp Prevention Trial that developed high-risk or advanced adenomas and were lower in participants consuming a flavonol-rich diet to which dry beans primarily contribute (Bobe et al., submitted). Similarly, serum IL-6 concentrations were elevated in obese, carcinogen-induced mice with pre-neoplastic lesions and were lower in mice fed the dry bean-rich diets (Mentor-Marcel et al., 2009). RNA concentrations of IL-6 in colon tissue were elevated in mice receiving the carcinogen and were lower in mice fed the dry bean-rich diets (Mentor-Marcel et al., 2009). Furthermore, we identified in a short-term human feeding study from fecal colonocyte microarray analysis sets of three genes that could be used as potential indicators of risk or exposure to dietary dry beans (Zhao et al., 2009).

150 Targeting Multiple Signaling Pathways With a Combination of Two Dietary Polyphenols, EGCG and Luteolin: Potential for Chemoprevention of Squamous Cell Carcinoma of the Head and Neck

A.R.M. Ruhul Amin, Dongsheng Wang, Johann Brandes, Fadlo R. Khuri, Zhuo Georgia Chen, **Dong Moon Shin**
Emory University

Tumorigenesis is a complex process involving interactions between multiple signaling pathways. Thus, targeting multiple signaling pathways with a combination of dietary compounds and other dietary compounds or existing molecularly targeted agents is gaining increasing attention. In the current study, we have found that two dietary polyphenols, epigallocatechin-3-gallate (EGCG) from green tea and luteolin from green vegetables, exhibited synergistic anti-tumor effects against a panel of cell lines established from squamous cell carcinoma of the head and neck (SCCHN). To elucidate the underlying mechanism of this combination, annexin V-PE staining and Western blotting were performed to study apoptosis and the expression patterns of cell-cycle and apoptosis regulatory proteins, respectively. A lentivirus-based expression system using short hairpin RNA (shRNA) was employed to ablate the expression of specific proteins. A nude mouse xenograft model was used to study the in vivo efficacy of the combined regimen. Treatment of cells with EGCG or luteolin alone induced minimal apoptosis (3–10%) in most of the cell lines tested. In contrast, simultaneous treatment with the combination of the two compounds tremendously increased apoptosis (60–80%) as evidenced by annexin V-PE staining and cleavage of PARP. Treatment with the two compounds also cleaved caspase-8 and -3 and induced the expression of DR5. Moreover, the expression of p53 and p73 and their transcriptional target p21 was increased after treatment with the compounds. Ablation of p53 with shRNA specific for p53 or inactivation of p73 with dominant negative p73 plasmid protected cells from apoptosis. In addition, combined treatment with the two compounds synergistically inhibited the expression of EGFR, the p65 subunit of NF-kappaB, its transcriptional target Bcl-2, and phosphorylation of AKT and STAT3. The combination of the two compounds more potently inhibited SCCHN cell line Tu212 xenograft growth in nude mice as compared with either single agent or control. Our results have, for the first time, identified that the combination of EGCG and luteolin has synergistic anti-tumor effects both in vitro and in vivo. These results suggest that the compounds induce apoptosis by activating p53 family members and inhibition of NF-kappaB and STAT3 pathways. Thus, the combination of EGCG and luteolin is highly promising for the further development of cancer prevention for SCCHN. (Supported by NIH/NCI awards P50CA128613, U01CA101244, and R01CA112643 for DMS).

151 Targeted Agent Intervention for Chemoprevention of Lung Cancer

Gerold Bepler, Jhanelle Gray, Mark Alexandrow, Soner Altiok, Frank Walsh
Thoracic Oncology Department, Moffitt Cancer Center, Tampa, FL

Background: Chemoprevention is crucial for reducing lung cancer mortality. The implementation and conduct of these trials is complex. We are summarizing our experience with an ongoing trial.

Methods: We are evaluating if enzastaurin, a protein kinase C beta inhibitor, decreases the Ki-67 labeling index in bronchial epithelium after 6 months of therapy compared to placebo. Eligibility includes age ≥ 45 years, smoking ≥ 30 pack year, quit > 1 year, and bronchial meta/dysplasia.

Results: Between December 2007 and May 2009, 1532 potential subjects were invited to participate by mail and at least three attempted telephone contacts. Phone contact was established with 1016 (66%) subjects: 456 (45%) declined, 466 (46%) were ineligible, and 94 (9%) were interested in and potentially eligible for trial participation. At the first visit, 85/94 (90%) were eligible and had an induced sputum exam. Cytology revealed that 54 (64%) had atypia, 29 were normal, and 2 were inadequate. Bronchoscopies with white light and laser-induced fluorescence and ≥ 3 biopsies have been done on 25/54 subjects (6 pending, 23 ineligible or withdrawn). Two had normal histology at all sites, 21 had metaplasia, and 2 had dysplasia in at least 1 site (92% abnormal bronchial histology rate). No occult cancers were identified.

Conclusions: Of 1532 preselected potential participants, 6.1% presented for a first face-to-face visit, 3.5% were eligible after a sputum exam, and 1.8% were fully eligible for randomization. Thus, to reach the accrual goal of 186 patients, we estimate that we will need to contact approximately 10,000 potential subjects. Our expectation had been to accomplish accrual over a period of 42 months from a pool of 3000 existing putative participants. Unless our eligibility rate increases, further sources of patients must be identified to accomplish our accrual goal.

152 Aspirin Use and Survival After Colorectal Cancer Diagnosis

Andrew T. Chan[1,2], Shuji Ogino[3], Charles S. Fuchs[2,3]

[1]Massachusetts General Hospital; [2]Brigham and Women's Hospital; [3]Dana-Farber Cancer Institute

Background: Aspirin reduces risk of colorectal neoplasia in randomized trials and inhibits tumor growth and metastases in animal models. However, the influence of aspirin on survival after diagnosis of colorectal cancer is unknown.

Methods: We examined the association between aspirin use after colorectal cancer diagnosis on colorectal cancer-specific and overall survival in a prospective cohort study of 1,279 men and women diagnosed with Stage I, II, or III colorectal cancer. Participants were enrolled in two health professional cohorts in 1980 and 1986, prior to diagnosis, and followed up through June 1, 2008.

Results: After a median followup of 11.8 years, there were 480 total deaths and 222 deaths due to colorectal cancer. The overall 5-year survival was 88% among those participants who used aspirin after colorectal cancer diagnosis and 83% among those who did not. Compared to non-users, participants who regularly used aspirin after diagnosis experienced a multivariate hazard ratio (HR) for colorectal cancer-specific mortality of 0.71 (95% CI, 0.53–0.95) and overall mortality of 0.79 (95% CI, 0.65–0.97). Among 719 participants who did not use aspirin before diagnosis, aspirin use initiated after diagnosis was associated with a multivariate HR for colorectal cancer-specific mortality of 0.53 (95% CI, 0.33–0.86). Among 459 participants with colorectal cancers that were accessible for immunohistochemical assessment, the effect of aspirin differed significantly according to COX-2 expression (Pinteraction=0.04). Regular aspirin use after diagnosis was associated with a lower risk of colorectal-cancer specific mortality among those whose primary tumors overexpressed COX-2 (multivariate HR 0.39; 95% CI, 0.20–0.76), whereas aspirin use was not associated with lower risk among those with primary tumors with weak or absent expression (multivariate HR 1.22; 95% CI, 0.36–4.18).

Conclusions: Regular aspirin use after the diagnosis of colorectal cancer is associated with lower risk of colorectal cancer-specific and overall mortality, especially among individuals with tumors that overexpress COX-2. These results suggest that aspirin may influence the biology of established colorectal tumors in addition to preventing their occurrence.

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153 Clinical Study of Resveratrol on Drug and Carcinogen Metabolizing Enzymes

H-H. Sherry Chow[1], Linda Garland[1], Chiu-Hsieh Hsu[1], Marjorie Perloff[2], James A. Crowell[3], and David Alberts[1]

[1]Arizona Cancer Center, The University of Arizona, Tucson, AZ; [2]Division of Cancer Prevention, [3]Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD

Resveratrol or 3, 4', 5-trihydroxystilbene was first identified in the dried roots of Polygonum cuspidatum, which is used in traditional Asian medicine for treatment of fungal infection, inflammation, hypertension, dermatitis, and hyperlipidemia. It is also found in several edible natural products such as grapes, peanuts, and berries, with up to 2 mg of resveratrol per serving. Resveratrol has been shown to inhibit carcinogenesis by affecting various molecular events in the initiation, promotion, and progression stages. The cancer chemopreventive activity of resveratrol has been demonstrated in vivo in a wide variety of tumors including skin, mammary, gastrointestinal, and liver cancer models.

Modulation of Phase I and Phase II enzymes has been suggested to be one of the mechanisms responsible for the cancer preventive effect of resveratrol. We conducted a clinical study to determine the effect of pharmacological doses of resveratrol on drug and carcinogen metabolizing enzymes. Forty-two healthy volunteers underwent baseline enzyme assessment following a minimum of 2 weeks of washout. For the enzyme assessment, blood lymphocyte glutathione S-transferase (GST) activity and GST-π level and serum total and direct bilirubin, a surrogate for UDP-glucuronosyl transferase (UGT) 1A1 activity, were measured. Baseline cytochrome P450 (CYP) enzyme activities were measured following the administration of a cocktail of CYP metabolic probe drugs. After the baseline evaluation, study participants took 1 gm of resveratrol once daily for 4 weeks. Enzyme assessment was repeated upon completion of the resveratrol intervention. Resveratrol intervention was found to suppress the activity of CYP3A4, 2D6, and 2C9. The geometric mean changes in the phenotypic index of 3A4, 2D6, and 2C9 were 33%, 70%, and 171%, respectively. Resveratrol intervention induced the CYP1A2 activity; the geometric mean change in the phenotypic index of 1A2 was 16%. The overall GST and UGT1A1 activity were minimally affected by the resveratrol intervention, while an induction of GST-π level and UGT1A1 activity was observed in individuals with low baseline enzyme level/activity. We conclude that high doses of resveratrol administration may modulate carcinogen activation, and potentially detoxification, but may lead to adverse drug-drug interactions.

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154 Anti-Stem Cell Factor Therapy to Maintain Normal Hematopoietic Progenitor Cell Function in Leukemia

Angela Colmone[1,2], Maria Amorim[1,3], Andrea Pontier[1], Elizabeth Jablonski[1,4], Sheng Wang[1], **Dorothy A. Sipkins**[1]

[1]The University of Chicago; [2]The American Journal of Pathology (currently); [3]The University of Pennsylvania (currently); [4]American Cancer Society, Illinois (currently)

Although defects in hematopoiesis are frequently observed in patients with malignant infiltration of the bone marrow (BM), the molecular bases of these phenomena are largely unknown. The potential consequences of cancer-mediated BM suppression are significant, including increased risk of infection, transfusion-dependence, and inability to tolerate maximal chemotherapy. In addition, it is relatively common that patients with residual BM disease show inadequate hematopoietic progenitor cell (HPC) mobilization into the peripheral circulation in response to cytokine treatment, precluding autologous bone marrow transplantation as a therapeutic option. Applying dynamic in vivo imaging to a mouse model, we show that leukemic cell growth disrupts normal hematopoietic progenitor cell (HPC) bone marrow niches and creates abnormal microenvironments that sequester transplanted human CD34+ (HPC enriched) cells. HPCs migrate into tumour niches in which the chemoattractant stem cell factor (SCF) is highly expressed. CD34+ cells in leukemic mice declined in number over time and failed to mobilize into the peripheral circulation in response to cytokine stimulation. Neutralization of stem cell factor (SCF) secreted by leukemic cells inhibited CD34+ cell migration into malignant niches, normalized CD34+ cell numbers, and restored CD34+ cell mobilization in leukemic mice. Finally, we determined whether changes in SCF expression could be detected in initial diagnostic BM samples from patients with pre-B ALL. Normal BM biopsies (no evidence of disease) and BM biopsies with known ALL involvement were assayed for SCF by immunohistochemistry of paraffin-embedded sections. Basal expression was low in 3/3 normal controls, while SCF staining was markedly elevated in 7/7 patient samples. These data suggest that the tumor microenvironment causes HPC dysfunction by usurping normal HPC niches and that therapeutic inhibition of HPC interaction with tumor niches may help maintain normal progenitor cell function in the setting of malignancy. Such an approach could decrease morbidity from cancer-related cytopenias, aid autologous stem cell collection, and improve outcomes in bone marrow transplantation for patients with active disease.

155 Colon Cancer Prevention With Selenium as Assessed by the Reduction in Aberrant Crypt Foci

Alan M. Diamond, Robert E. Carroll, Richard V. Benya

University of Illinois at Chicago, IL

Selenium is an essential trace element that has received considerable attention as a possible means of reducing cancer incidence. There is a substantial body of evidence indicating that low level, non-toxic supplementation of the diet with selenium can reduce cancer frequencies in rodent models. In humans, there is epidemiological data supporting the inverse association between selenium intake and cancer incidence for several organ types, including the prostate and colon. In addition, positive data was obtained from the National Prevention of Cancer (NPC) trial indicating that selenium provided as selenized yeast at a daily dose of 200 ug/day may reduce the incidence of colon and prostate cancer in certain individuals. In contrast, the results of large prostate cancer prevention trial SELECT indicated that selenium provided at the same 200 ug/day dose, but in the form of selenomethionine, was ineffective in reducing prostate cancer incidence in that study's population.

The search for supplements that would reduce colon cancer risk would benefit from examination of biological endpoints that would serve as surrogates for colon cancer, such as aberrant crypt foci (ACF), preneoplastic lesions that have been a useful biomarker for colon carcinogenesis in rodent model systems. Initial data have indicated an inverse association between dietary intake of selenium and ACF frequency in humans. The planned study is designed to investigate whether selenium provided to individuals at 200 ug/day as selenized yeast for 6 months can reduce the number of ACF lesions in patients as compared to those receiving placebo. Allelic identity of several genes will be determined to assess whether variations within the coding sequence of these genes can influence the efficacy of selenium. Among the genes to be examined will be that encoding the cytosolic form of glutathione peroxidase, GPx-1. The levels of GPx-1 have been shown to influence DNA damage susceptibility in cell culture studies, and functional GPx-1 polymorphisms are associated with the risk of cancer of several organ types. Collectively, these studies are intended to establish the usefulness of examining ACFs as a biomarker for colon cancer prevention studies with selenium and to identify subpopulations that would benefit from selenium supplementation.

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156 Mechanisms for the Chemoprevention of Cancer

Raymond N. DuBois[1], Dingzhi Wang[1], Andrew Dannenberg[2], Timothy Hla[3], Sudhansu K. Dey[4]

[1]The University of Texas M. D. Anderson Cancer Center, Houston, TX; [2]Weill Cornell Medical College, New York, NY; [3]University of Connecticut Health Science Center, Center for Vascular Biology, Farmington, CT; [4]Cincinnati Children's Medical Center, Cincinnati, OH

Solid malignancies such as breast, cervical, ovarian, and colon cancer cause a significant number of deaths each year. These malignancies often involve the upregulation of pro-inflammatory mediators and cyclooxygenase (COX) enzymes, generating bioactive lipids that promote carcinogenesis. These enzymes are the primary targets for non-steroidal anti-inflammatory drugs (NSAIDS). This Program Project Grant (PPG) is based upon the hypothesis that COX-derived prostaglandins (PGs) play a significant role in the development of solid malignancies. Our Program Project is also focused on determining the molecular mechanisms responsible for the cancer chemopreventive effects of NSAIDS. The long-term use of COX-2 inhibitors is associated with increased risk of cardiovascular complications in certain subpopulations. This highlights the need to reveal the downstream mechanisms that drive carcinogenesis. Identifying downstream targets is critical to developing novel approaches that maximize the anti-cancer benefit while reducing cardiovascular side effects. This research has added considerably to understanding the role of prostaglandins in malignancy. Insights gained from this PPG provide the basis for alternate strategies to either suppress tissue levels of PGs or inhibit their actions. These studies center upon four highly successful projects: (1) Understanding the role of COX downstream signaling pathways in cancer, (2) COX-2: a target for the prevention of cervical cancer, (3) COX-2 regulation and function in tumor biology, and (4) COX-1: a target for ovarian cancer prevention and treatment. The outstanding success of these projects revolves around three very well integrated cores. Core A consists of a highly experienced leadership team that facilitates synergistic exchange between all projects and cores. Core B performs sophisticated analytical characterization of bioactive lipids isolated from complex biological matrices. Core C provides members of the PPG with centralized resources for the development, maintenance, distribution and cryopreservation of numerous mouse models. Our team of investigators has pioneered numerous concepts regarding the role of eicosanoids in cancer, resulting in numerous peer-reviewed publications in high-ranking journals. These projects integrate outstanding core facilities and support staff, robust collaborations, extensive clinical and basic science expertise, and large number of shared resources at multiple institutions. Our goal is to set new milestones for the chemoprevention of solid malignancies to reduce the overall cancer burden.

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157 Flaxseed Lignan for Chemoprevention in Premenopausal Women at High Risk for Development of Breast Cancer

Carol J. Fabian, Qamar J. Khan, Priyanka Sharma, Susan Baxa, Trina Metheny, Carola M. Zalles, Theresa Phillips, Bruce F. Kimler

University of Kansas Medical Center, Kansas City, KS

The lignans enterolactone and enterodiol are derived from the action of gut bacteria on ingested Secoisolariciresinol diglycoside (SDG), which is commonly found in flaxseed. Enterolactone and enterodiol are thought to impair mammary carcinogenesis via reduction in aromatase activity and the mid-cycle surge of luteinizing hormone. We assessed the modulatory activity of 1 year of SDG on a number of risk biomarkers for breast cancer in a prospective Phase II pilot study. The primary endpoint was a change in proliferation in benign breast tissue as measured by Ki-67 immunocytochemistry. Pre-menopausal women age 21–55 at increased risk for breast cancer underwent a baseline random periareolar fine needle aspiration (RPFNA) between the first and 10th days of their menstrual cycle. Those with RPFNA evidence of hyperplasia and Ki-67 $\geq$ 2% were invited to participate. Women taking flaxseed or oral contraceptives were ineligible. All women took one Brevail® (Lignan Research) capsule containing 50 mg of SDG daily. Ki-67 staining was performed with DAKO M7240 antibody on hematoxylin counterstained slides, and the number of cells staining positive in 500 cells within hyperplastic clusters was counted. Forty-nine women were enrolled on study between February 2006 and June 2008. Of these, 4 stopped prematurely and 45 (92%) have completed study and undergone follow-up RPFNA to provide evaluable specimens. Baseline characteristics of the 45 women completing study are as follows: median age 43 (range 29–51), median baseline 5-year Gail model risk 1.6% (range 0.1–5.7%), median Ki-67 4.0% (range 2.0–16.8%). Some 35 % had cytologic evidence of hyperplasia without atypia and 62% had atypia. At repeat RPFNA, Ki-67 expression was reduced, median value of 2.0%, range 0–15.2%, and median relative reduction of 0.67. Thirty-six of the 45 women (80%; $p<0.001$ by Wilcoxon signed ranks test) demonstrated a decrease in proliferation. The reduction in proliferation as measured by Ki-67 expression in hyperplastic benign breast tissue after 12 months of 50 mg of SDG administered daily as Brevail® warrants study in a randomized, blinded, placebo-controlled clinical chemoprevention trial.

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158 Efficacy of Chemopreventive Agents in a Urinary Bladder Cancer Model: Study Design and Endpoints

Clinton J. Grubbs[1], Vernon E. Steele[2], and Ronald A. Lubet[2]
[1]University of Alabama at Birmingham; [2]National Cancer Institute

Studies were performed to determine the most predictive and relevant data for clinical trials for the prevention of urinary bladder cancer. Various chemopreventive agents and different treatment regimens were tested using the hydroxybutylbutylnitrosamine OH-BBN induced urinary bladder cancer model in female Fischer-344 rats. In all studies, OH-BBN was given by gavage 2x/week for 8 weeks at a dose of 150 mg/treatment. Rats were treated with naproxen 400 mg/kg diet, Iressa 10 mg/kg BW/day, resveratrol 1000 mg/kg diet, or aspirin 300 or 3000 mg/kg diet employing one of three protocols: A treatment beginning one week after OH-BBN and continuing for 7 months; B treatment beginning 3 months after OH-BBN when microscopic lesions already existed and continuing for 3 months; C treatment beginning one week after OH-BBN and continuing for 4 months. In protocols A and B, the weight of the bladder lesions and the occurrence of large tumors were primary endpoints; while in Protocol C microscopic cancers were the endpoints. Using protocol A, naproxen, Iressa, resveratrol, low dose aspirin, and high dose aspirin altered the formation of large bladder cancers by 87↓, 90↓, 3↑, 6%↓, and 60%↓, respectively. Using protocol B, Iressa and naproxen were also highly effective. Protocol C evaluation revealed that only Iressa caused significant decreases in microscopic bladder cancers, 63%↓. In summary, Iressa and naproxen protocols A and B were highly effective in preventing development of urinary bladder cancers. Low dose aspirin and resveratrol were ineffective. Only Iressa was effective using protocol C. The question of when to initiate exposure to a chemopreventive agent when screening compounds in an animal model is still debatable. However, it is felt that starting later in the cancer progression process rather than at the time of carcinogen treatment or very early thereafter is preferable given the nature of the Phase III clinical trials presently being performed. Again, the present data indicate that naproxen, which has an excellent cardiovascular profile or EGFR inhibitors, might be effective in an adjuvant prevention setting.

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159 Potent Chemoprotective Activity of Apiaceae Spices

Ramesh C. Gupta, Jeyaprakash Jeyabalan, Farrukh Aqil, Manicka V. Vadhanam, David J. Schultz and Srivani Ravoori
University of Louisville, Louisville, KY

The Apiaceae spices, namely ajowan, angelica, anise, caraway, carrot seeds, celery, coriander, cumin, dill, fennel ,and parsley, were tested for their chemopreventive potential against estrogen-mediated breast cancer. 4-Hydroxy-17ß-estradiol (4E2) was incubated with DNA and Cu2+ in the presence and absence of aqueous and acetonitrile extracts of the spices. Analysis of oxidative DNA adducts by 32P-postlabeling showed that aqueous extracts inhibited the adduct formation substantially (83–97%). Non-aqueous extracts were largely ineffective. When extracts of anise, caraway, coriander, cumin, dill, and fennel were tested in microsomal activation of estradiol/Cu2+, both the aqueous and non-aqueous extracts were equally effective in inhibiting the adduct formation. These data suggest the presence of two sets of phytochemicals in test spices: (i) water-extractable compounds that acted as antioxidants and (ii) acetonitrile-extractable compounds that presumably inhibited the conversion of E2 to 4E2. Their in vivo chemopreventive potential was determined using estrogen-mediated ACI rat mammary tumorigenesis model. Groups of animals were provided AIN 93M diet or diets supplemented with cumin and fennel (2.5%, w/w) and were then challenged with a 17ß-estradiol implant two weeks later. Analysis of mammary cell proliferation by measurement of PCNA and Ki-67 three weeks later showed significant antiproliferative activity, with fennel diet being more effective. Continuation of the study with fennel showed a delayed tumor latency (15 versus 18 weeks) and lower tumor incidence (14% versus 33%) compared with control diet. However, at the end of 29 weeks, there was no statistical difference in tumor incidence, tumor burden, and tumor multiplicity. Further studies with higher doses of cumin, fennel, and ajowan (5% and 7.5%) all resulted in a dose-dependent inhibition of (i) estrogen-mediated mammary cell proliferation, (ii) expression of mammary CYP1B1 protein, and (iii) the plasma prolactin, a key factor in mammary tumorigenesis. The spice treatments did not affect the diet intake and the body weight, indicating that the spices were well tolerated. Together, our data indicate that the Apiaceae spices are highly protective against estrogen-mediated mammary cell proliferation, P450 induction, and plasma prolactin.

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160 Oral Iloprost Improves Endobronchial Dysplasia in Former Smokers

Robert L. Keith[1,2], John K. Kittelson[2], Patrick J. Blatchford[2], Mary Jackson[2], Mark W. Geraci[2], Wilbur A. Franklin[2], Fred R. Hirsch[2], John Minna[3], Paul A. Bunn[2], York E. Miller[1,2]

[1]Denver Veterans Affairs Medical Center; [2]University of Colorado Comprehensive Cancer Center; [3]Hamon Center for Therapeutic Oncology, University of Texas Southwestern

Tissue microarray studies have shown that the majority of NSCLC have decreased expression of prostacyclin synthase (PGIS). Prostacyclin supplementation prevents development of lung cancer in multiple murine models, including cigarette smoke exposure. Based on these promising results, a multi-center, double-blind, placebo-controlled phase II trial of iloprost in subjects at increased risk for lung cancer was recently completed. Subjects were selected for the trial if they met the following criteria: current or former smoker (≥ 20 pack years tobacco exposure); at least mild cytologic atypia on sputum cytology or a history of endobronchial dysplasia; and no previous history of cancer. Autofluorescence and white light bronchoscopy were performed with 6 standard endobronchial sites biopsied along with all other visually abnormal areas. Subjects were then randomized to oral iloprost or placebo for 6 months, followed by a second bronchoscopy with repeat biopsy of all areas sampled during the first bronchoscopy. The primary endpoints for the study are bronchial histology. The enrollment goal of 152 subjects was met, of which 125 completed the trial. Baseline characteristics showed the subjects to be matched in terms of age, tobacco exposure, and sputum cytology. All endobronchial biopsies were scored on a 1–8 scale based on WHO criteria. Of the 152 enrolled subjects, 74% (113/152) had at least one mildly dysplastic or worse biopsy. Endobronchial histology was summarized using three distinct measures: worst biopsy score (Max), dysplasia index (DI - the % of biopsies with a score of ≥ 4 (mild dysplasia)), and average of all biopsy scores (Avg). At the baseline bronchoscopy, current smokers had a significantly higher Avg score than former smokers (3.0 vs. 2.1, $p<0.001$). There were no other significant differences between treatment groups in baseline histologic measures. Follow-up bronchoscopy was performed on 125 subjects (60 - iloprost, 65 - placebo). Former smokers treated with iloprost showed significant improvement in all histology measures: Max (iloprost vs. placebo: –0.59 vs. 0.50, $p = 0.004$), DI (–8.84 vs. 0.10, $p = 0.019$) and Avg (–0.33 vs. –0.02, $p = 0.046$). Iloprost did not have any significant effects in participants who continued to smoke during the trial. The iloprost chemoprevention trial has been completed, and our recruitment model led to a high percentage of subjects with endobronchial dysplasia. Former smokers who received iloprost showed a statistically significant improvement in all histologic measurements. Based on the results of our Phase II trial, oral iloprost should progress to a larger phase III trial.

161 Gender Differences in Long-Term Dexrazoxane Cardioprotection in Doxorubicin-Treated Children With Acute Lymphoblastic Leukemia

Steven E. Lipshultz[1], Rebecca E. Scully[1], Stuart R. Lipsitz[2], Stephen E. Sallan[3], Lewis B. Silverman[3], Tracie L. Miller[1], E. John Orav[2], Steven D. Colan[4], the Dana-Farber Cancer Institute Acute Lymphoblastic Leukemia Consortium

[1]University of Miami Miller School of Medicine, Miami, FL; [2]Brigham and Women's Hospital, Boston, MA; [3]Dana-Farber Cancer Institute, Boston, MA; [4]Children's Hospital Boston, Boston, MA

Doxorubicin (DOX) causes progressive cardiac dysfunction in children being treated for acute lymphoblastic leukemia (ALL). One proposed mechanism of DOX-induced cardiac damage is the formation of DOX-iron complexes, which can generate reactive oxygen species capable of intracellular damage and cell death. During Dana-Farber Cancer Institute Protocol 95–01, adding the iron chelator dexrazoxane (DEX) prior to DOX treatment resulted in reduced cardiac injury in children being treated for ALL. In order to assess the long-term cardiac status of these patients, we centrally remeasured echocardiograms from childhood high-risk ALL survivors in their first continuous remission who were randomly assigned to treatment with DOX (n = 66; 30 mg/m2/dose for 10 doses) or DOX plus DEX (n = 68; 300 mg/m2/dose). Demographics and median followup (DOX 5.3 vs DEX/DOX 5.5 y) were similar in both arms. Our follow-up studies showed mean LV end systolic dimension (ESD) z-score was significantly larger than predicted by body-surface area for patients receiving DOX (mean = 0.46, P-value vs. normal = 0.01) but not for DEX/DOX (mean = 0.06, P = 0.74); DOX LV fractional shortening (FS; –0.78, P = .001; DEX/DOX = –0.38, P = 0.11) and thickness to dimension ratio (TD; –0.96, P < .001; DEX/DOX = –0.32, P = 0.08) were also abnormal. In both groups, LV end diastolic posterior wall thickness (EDPWT) was reduced, though more so for DOX (–1.19, P < .001) than DEX/DOX (–0.74, P < .001). By gender, LVESDz was significantly larger than normal for DOX boys (mean = 0.48, P = 0.04) but not DEX/DOX boys (0.19, P = 0.41) or girls (DOX girls = 0.38, P = 0.22; DEX/DOX girls = –0.17, P = 0.56). LVFSz was significantly different from normal in DOX girls (mean = –1.29, P < .001) but not DEX/DOX girls (–0.22, P = 0.54) or boys (DOX = –0.45, P = 0.15; DEX/DOX = –0.52, P = 0.09), as was LVTDz (DOX girls = –1.03, P < .001; DEX/DOX girls = 0.02, P = 0.93). DEX/DOX girls were the only group with a normal LVEDPWTz (mean = –0.43, P = 0.07; DOX girls = –1.43, P < .001; DOX boys = –1.05, P < .001; DEX/DOX boys = –0.94, P < .001). Our data show DEX is correlated with less LV dilation and remodeling, suggesting a cardioprotective effect. Similarly, LV structure and function are more normal among children who received DEX. Further, girls show worse DOX effects and greater DEX cardioprotection. The finding that girls are significantly more responsive to DEX than boys suggests that, by interrupting DOX-iron complexes, DEX is significantly more likely to benefit girls than boys.

162 Lung Cancer Chemoprevention With Celecoxib in Ex-Smokers

Jenny T. Mao[1,2], Michael D. Roth[1], Michael C. Fishbein[1], Zuo-Feng Zhang[1], Jian Yu Rao[1], Denise Aberle[1], Carmack E. Holmes[1], Donald P. Tashkin[1], Lee Goodglick[1], Eva Szabo[3], Steven M. Dubinett[1], Robert Elashoff[1], David Elashoff[1]

[1]University of California, Los Angeles; [2]New Mexico VA Healthcare System and University of New Mexico; [3]National Cancer Institute

Preclinical data suggest that the cyclooxygenase-2 (COX-2)/prostaglandin-E2 (PGE2) pathway plays a pivotal role in carcinogenesis. Overproduction of PGE2 occurs in the setting where COX-2 expression is upregulated and is associated with a variety of carcinogenic mechanisms. Results from a pilot phase IIa trial in high-risk smokers suggested that Celecoxib (a COX-2 inhibitor) might reduce PGE2 production and restore anti-tumor immunity in the lung microenvironment, and reduce proliferation indices (Ki-67 labeling index, KI-67 LI) on the bronchial epithelium. These data supported the antineoplastic effect of COX-2 inhibitors and provided the rationale for evaluating their potential in lung cancer chemoprevention. Funded by a U01 mechanism, a phase IIb, randomized, placebo-controlled, crossover pilot study was carried out to determine the feasibility of Celecoxib for lung cancer chemoprevention in ex-heavy smokers (age > 45, > 30 pack years of smoking history and at least one year of smoking cessation). Qualified participants underwent comprehensive screening with low-dose helical CT scan and fluorescence bronchoscopy. Celecoxib (400 mg twice daily) was evaluated for its impact on cellular and molecular events associated with lung carcinogenesis. We prescreened 4,470 subjects, actively screened 323 subjects, performed screening bronchoscopy on 142 subjects, and randomized a total of 137 subjects, of which 101 subjects were evaluable. The primary endpoint of the study was modulation of the Ki-67 LI on bronchial mucosa following 6 months of treatment. The aggregate mean change in Ki-67 LI for each subject was determined by averaging the change score (pre-treatment vs. post-treatment) for all evaluable biopsies from that subject. Modulation of Ki-67 LI in response to treatment was then analyzed by comparing these aggregate mean changes between the treatment versus placebo groups. Primary analysis indicates that 6 months of Celecoxib treatment significantly decreased Ki-67 LI in heavy former smokers by an average of 34%, similar to what was observed in active smokers in the phase IIa study, whereas an average of a 4% increase was observed in the placebo group ($p = 0.04$; t test). A significant treatment effect based on the interaction between treatment and baseline expressions on Ki-67 LI was also observed using a mixed-effects analysis ($p = 0.006$). Evaluations of a variety of secondary surrogate endpoint biomarkers are currently underway.

163 Proteomic Approaches for Development of Chemoprevention Targets

Masako Nakanishi[1], Antoine Menoret[2], Anthony Vella[2], Shingo Miyamoto[1], **Daniel W. Rosenberg**[1]

[1]Center for Molecular Medicine and Neag Cancer Center, University of Connecticut Health Center, Farmington, CT; [2]Department of Immunology, University of Connecticut Health Center, Farmington, CT

A complex interplay between genetic background and environmental factors is likely to influence the efficacy of intervention strategies for colon cancer prevention. One approach to optimizing cancer chemoprevention is to identify individuals who will be responsive to a particular agent. This may, in principle, be accomplished using genetic approaches, provided that genes important for a given biological response may be identified. On the other hand, a proteomic approach offers a number of distinct advantages, including the ability to detect post-translational modifications (PTMs) of proteins that play a fundamental role in generating a positive response (e.g., tumor suppression).

In the following study, we describe the development of a proteomic-based approach to identifying tumor changes in real-time in response to chemoprevention treatment. It will be possible to determine which subpopulations of early colon lesions develop into tumors and whether chemoprevention agents suppress the rate of adenoma formation or promote their regression. Our method is predicted to recapitulate potential clinical scenarios, in which protein markers can be used to identify individuals with high-risk adenomas. In addition, our long-term goal is to customize chemoprevention in human populations based on expression of predictive proteins or genes uncovered in precancerous lesions.

Our initial approach has been to characterize the response of ApcΔ14/+ mice to sulindac, a commonly used chemoprevention agent that has varying efficacy. We have adapted this strategy resveratrol and black raspberry powder. In addition, we anticipate that our refinements in lesion imaging and feature recognition within the topography of the colon, established from mouse chromoendoscopy, may eventually be translated into the clinic as a procedure for monitoring the precise location of lesions that can then be followed longitudinally over time.

164 Fat Water Ratio and Diffusion-Weighted MRI Applied to the Measure of Breast Density as a Drug Response Biomarker for Chemoprevention Studies

Patricia A. Thompson[1,2], Veronique Poulin[1,2], Maria Altbach[1], Matthew Kupinski[1], Denise Roe[1,2], Theodore Trouard[1], Per Granstrom[1], Georgette Frey[1,2], Scott Squire[1], Alison T. Stopeck[1,2]

[1]University of Arizona,Tucson, AZ; [2]Arizona Cancer Center,Tucson AZ

In spite of strong preclinical evidence for a number of promising prevention agents for the breast, advances in breast cancer chemoprevention are limited by the lack of a quantifiable and non-invasive intermediate biomarker that informs on agent response and risk modulation. Results from the IBIS-1 trial of tamoxifen (TAM) provide the first evidence that the cancer prevention activity of TAM is mediated, at least in part, through effects on mammographic density. Low precision with poor reproducibility, sensitivity, and accuracy, however, impede the use of mammographically-determined density as a drug response biomarker for screening novel agents.

We used a novel MRI radial GRAdient and Spin-Echo (radial-GRASE) MRI pulse sequence in combination with a fat-water decomposition algorithm to obtain fat-water ratios (FWR) with correction for the effect of field inhomogeneities. The method is referred to as FWR-MRI. In addition, we collected diffusion-weighted MR images (DW-MRI). The acquisition time for the FWR-MRI and DW-MRI sequences was less than 5 minutes, and contrast was not utilized. The costs for all required MR scans approximated those for standard mammography. A total of 25 women were studied, including 8 with no history of breast cancer, 15 with breast cancer, and 2 at high risk for breast cancer. All subjects were ascribed a BI-RAD score and % density based on mammogram.

Histograms of subjects with less-dense breasts were single peaked, while the histograms from subjects with dense breasts were dual-peaked. We chose initially to look at the fraction of pixel values (Frc) below 30, 40, and 50% fat. For Frc 30, 82% of the variance was between subjects, 11% was between-sides within the same subject, and 7% was between-replicates within the same side and subject. The intraclass correlation coefficient was found to be highest for Frc 30. Highly significant relationships ($p < 0.001$) were observed for all three measures of FWR-MRI (Frc 30, Frc 40, Frc 50) and the mean apparent diffusion coefficient (ADC) values, % density by mammography ($p < 0.001$), and mammographic BI-RADS score ($p < 0.001$). Analysis of data obtained over the entire breast was more predictive than assessment of only the largest slice (in terms of volume). Menopausal status (post versus pre) was strongly related to the FWR-MRI values ($p < 0.001$).

These data illustrate the utility of FWR-MRI and DW-MRI to accurately measure breast density. Studies are ongoing to determine if early changes in FWR or DW-MRI can be observed in response to chemoprevention agents.

165 Suppression of Mammary Carcinogenesis in MMTV-neu Mice by Dietary Benzyl Isothiocyanate Administration

Renaud Warin, William H. Chambers, **Shivendra V. Singh**

Departments of Pharmacology & Chemical Biology and University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

Breast cancer is a leading cause of cancer-related deaths in women worldwide. Therefore, agents that are relatively safe but can inhibit onset and/or progression of this malignancy are highly desirable. In the present study, we tested chemopreventive efficacy of benzyl isothiocyanate (BITC), a constituent of many edible cruciferous vegetables, on mammary carcinogenesis using MMTV-neu mice. The control mice (8 weeks of age) were placed on AIN-76A diet for 25 weeks, whereas the experimental groups of mice (8 weeks old) were fed AIN-76A diet supplemented with 1 or 3 mmol BITC/kg diet. Initial as well as final body weights of the mice fed 3 mmol BITC/kg diet was modestly but significantly lower compared with control or 1 mmol BITC/kg group. However, the BITC administration did not cause any toxicity as judged by histopathology of vital organs including liver, lung, kidney, and heart. The average food consumption was slightly higher in the 3 mmol BITC/kg group compared with control or 1 mmol BITC/kg group. Cumulative incidence of abnormal structures (hyperplasia, carcinoma in situ, and adenocarcinoma) was about 33% and 28% lower ($P= 0.011$ by Fisher's test), respectively, in the breast of 1 and 3 mmol BITC/kg groups compared with control mice. BITC at both doses exhibited inhibition of hyperplasia incidence (37–43 % inhibition compared with control) and burden (>70% lower compared with control). The incidence of mammary carcinoma was significantly lower in mice fed 3 mmol BITC/kg diet compared with control mice ($P= 0.067$ by Fisher's test). The tumor sections from BITC-fed mice displayed reduced cell proliferation (Ki-67 expression), increased apoptotic bodies (TUNEL staining), increased expression of E-cadherin, and infiltration of CD3+ T cells. Even though BITC treatment increased cytotoxicity of NK cells in vitro, dietary feeding of BITC failed to augment NK cell lytic activity in an ex vivo assay. In conclusion, the results of the present study provide strong impetus to determine efficacy of BITC for prevention of mammary cancer in humans.

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166 A Phase I Trial of ALA PDT for Treatment of Oral Leukoplakia

Stuart J. Wong[1], Bruce Campbell[1], Becky Massey[1], Denis Lynch[2], Ezra E. W. Cohen[3], Borko D. Jovanovic[4], Raymond C. Bergan[4]

[1]Medical College of Wisconsin; [2]Marquette University School of Dentistry; [3]University of Chicago Cancer Research Center; [4]Lurie Cancer Center Northwestern University

Leukoplakia is a premalignant condition involving the oral mucosa that is characterized by a white patch or plaque and coexists with varying grades of dysplasia. Oral leukoplakia is an excellent clinical model for examining cancer prevention strategies. Photodynamic therapy (PDT) involves the topical or systemic administration of a photosensitizing agent that, in the presence of light corresponding to an optimal wavelength, creates reactive oxygen species capable of inducing cytotoxic damage. Aminolevulinic acid (ALA) is an endogenous metabolite that, when exogenously administered, bypasses the negative feedback control of its metabolic pathway and leads to preferential accumulation of the photosensitizer protoporphyrin IX in mucosal tissues rather than the underlying stroma. We hypothesize that use of ALA PDT for oral leukoplakia is safe and tolerable and that quantitative histologic assessment of response using specific biologic markers (DNA ploidy, proliferation using Ki-67, apoptosis using TUNEL, cyclin D1, p53) is feasible. To test this hypothesis we initiated a phase I clinical trial to determine the toxicity and feasibility of ALA PDT, to assess treatment efficacy by examining clinical and quantitative histologic response, and to explore the association of response with specific molecular and biologic markers. The study examines six cohorts in which escalating doses of long pulse dye laser from 2 to 12 J/cm2 (585 nm light, Photogenica SV pulsed dye laser system) are administered with a fixed dose of oral ALA (30 mg/kg). The ALA dose was reduced from 60 mg to 30 mg after a subject in cohort 1 experienced a grade 3 event due to transient liver function test elevation. Enrollment into this trial is ongoing.

167 Discovery and Validation of Molecular Targets, Biomarkers and Non-Toxic Dietary Interventions for Cancer Prevention

Matthew R. Young[1], Gerd Bobe[1,2], Roycelynn Mentor-Marcel[1,2], Terry Hartman[3], Robb Chapkin[4], Elaine Lanza[1,5], John Milner[6], Young Kim[6], **Nancy H. Colburn**[1]

[1]Laboratory of Cancer Prevention, Center for Cancer Research, National Cancer Institute (NCI); [2]Preventive Oncology Program, NCI; [3]Pennsylvania State University; [4]Texas A & M University; [5]Cytonix; [6]Division of Cancer Prevention, NCI

The Gene Regulation Section, Laboratory of Cancer Prevention (LCP), Center for Cancer Research, National Cancer Institute is focused on understanding the early events in tumorigenesis with the hope of finding new molecular targets, biomarkers, and non-toxic interventions that will prevent or significantly delay cancer. Our work in vitro and with genetically engineered mice (GEM) identified the transcription factor AP-1 as a molecular target for cancer prevention (Young et al., 2003). Mice that express dominant negative c-Jun (TAM67) are protected from environmental and oncogene induced skin and breast cancer without any noted toxic side effects (Young et al., 2003, Shen et al., 2008). A high throughput screen has identified novel natural compounds that target AP-1 and/or NFκB without inhibiting cell proliferation or survival (Ruocco et al., 2007, Kang et al., 2009).

The LCP has established multiple collaborations to identify dietary factors that prevent cancer. In collaboration with Michigan State University, Pennsylvania State University, and Texas A&M University, we have been identifying potential molecular targets and biomarkers of efficacious response to dry bean-based diets in parallel in human dietary intervention trials and mouse models of colon carcinogenesis. These studies are based on our observation that high dry bean intake decreased advanced colorectal adenoma recurrence in humans (Lanza et al., 2006) and protected against chemically induced colon carcinogenesis in obese mice (Bobe et al., 2008). So far, IL-6 has been identified as a potential biomarker and molecular target. Serum IL-6 concentrations were elevated in participants of the Polyp Prevention Trial that developed high risk or advanced adenomas and were lower in participants consuming a flavonol-rich diet to which dry beans primarily contribute (Bobe et al., submitted). Similarly, serum IL-6 concentrations were elevated in obese, carcinogen-induced mice with pre-neoplastic lesions and were lower in mice fed the dry bean-rich diets (Mentor-Marcel et al., 2009). RNA concentrations of IL-6 in colon tissue were elevated in mice receiving the carcinogen and were lower in mice fed the dry bean-rich diets (Mentor-Marcel et al., 2009). Furthermore, we identified in a short-term human feeding study from fecal colonocyte microarray analysis sets of three genes that could be used as potential indicators of risk or exposure to dietary dry beans (Zhao et al., 2009).

168 Wnt/beta-Catenin Inhibitors for Colon Cancer Chemoprevention and Therapy

Wen Zhang[1], Xi Chen[1], David Watt[2], B. Mark Evers[1,3], **Chunming Liu**[1,2]

[1]Markey Cancer Center, Departments of [2]Molecular and Cellular Biochemistry, and [3]Surgery, University of Kentucky, Lexington

Colorectal cancer is the second leading cause of cancer-related mortality in the United States and accounts for more than 56,000 deaths per year. More than 80% of colorectal cancers have mutations in the APC (Adenomatous Polyposis Coli) gene, and about 13% of colorectal cancers have mutations in the CTNNB1 gene (beta-catenin). Mutations in either gene stabilize beta-catenin and activate Wnt signaling, ultimately leading to cancer. Our previous studies provide insights into the molecular mechanisms of how beta-catenin degradation is regulated and why mutations of APC and beta-catenin cause cancers. Since a mutation in APC or beta-catenin is the initiating step of colon carcinogenesis, the Wnt pathway is a very attractive target for designing chemopreventive and therapeutic agents. Wnt signaling can be inhibited at multiple levels. However, because of APC and beta-catenin mutations, agents for colorectal cancer must block the function of beta-catenin in the nucleus. We have demonstrated that KLF4, a novel beta-catenin inhibitor in the nucleus, can repress tumorigenesis of colon cancer cells containing APC or beta-catenin mutation. In this study, we are developing small molecular inhibitors targeting Wnt/beta-catenin pathway. We have established a stable colon cancer cell line, LS174-TopGFP. In this cell line, GFP is control by seven copies of TCF-binding element. The GFP protein contains a PEST domain for degradation; the half-life of GFP-PEST is 2h. Since LS174 cell contains a beta-catenin mutation and has constitutively active Wnt signaling, we can detect strong GFP signal in these cells. When Wnt signaling was inhibited by KLF4 or other inhibitors, GFP signal was significantly reduced within 12h. We are using this assay to screen and characterize novel Wnt/beta-catenin inhibitors. We have identified several Wnt/beta-catenin inhibitors from natural compounds including food components. These agents can inhibit TopFlash reporter, an indicator for Wnt/beta-catenin activation. They also inhibited the expression of endogenous Wnt/beta-catenin target genes, such as c-myc, cyclinD1, and survivin, in colon cancer cells. In addition, they induced expression of cell cycle inhibitor, p21, and inhibited colon cancer cell proliferation. By chemical modification, we have identified several key groups of these compounds. We are investigating the mechanisms of these agents in Wnt/beta-catenin inhibition. We are collaborating with Dr. David Watt to design and synthesize novel agents and collaborating with Dr. Mark Evers to test these agents in vivo.

169 Targeting Signaling Pathways in Pre-Clinical Studies Using Mouse Models of Prostate and Bladder Cancer

Cory Abate-Shen, Carolyn W. Kinkade, Catherine Seager, Anna Puzio-Kuter, Mireia Castillo-Martin, Trushar Patel, Alvaro Aytes Meneses, Mariya Rozenblit, Edward Gelmann, Michael M. Shen, James McKiernan, and Carlos Cordon-Cardo

Departments of Urology, Pathology and Cell Biology, Medicine, and Genetics & Development, Columbia University Medical Center, Herbert Irving Comprehensive Cancer Center, New York, NY

Our laboratories have been investigating new approaches for treatment of prostate and bladder cancer by integrating analyses of genetically-engineered mutant (GEM) mice with clinical and functional analyses of human tumors. In our analyses of prostate cancer, we have generated GEM mice based on loss-of-function of the Nkx3.1 homeobox gene and the Pten tumor suppressor gene, which recapitulate the spectrum of disease progression including castration-resistant prostate cancer. Based on previous studies showing that the Akt/mTOR and Erk MAP pathways cooperate in prostate cancer progression, we performed pre-clinical studies in these GEM mice to examine the consequences of combinatorial inhibition of these signaling pathways for tumorigenesis. We found that combination therapy using Rapamycin, an inhibitor of mTOR, and PD0325901, a MEK inhibitor, is potently anti-tumorigenic, particularly in androgen-independent contexts. Furthermore, these signaling pathways are coordinately deregulated in human prostate cancer. We have proposed that combination therapy targeting the Akt/mTOR kinase and Erk Map kinase signaling pathways may be effective for treatment of advanced prostate cancer.

In our analyses of bladder cancer, we have generated a new GEM model by targeted gene deletion in bladder epithelium using an adenovirus expressing Cre recombinase. We have found that combinatorial inactivation of p53 and Pten leads to invasive bladder cancer. We have further shown that combinatorial inactivation of p53 and PTEN in human cells promotes bladder tumorigenesis in a renal grafting model, while their combined alteration in human bladder tumors is correlated with poor survival. The synergistic consequences of p53 and Pten inactivation in bladder tumors are mediated in part by deregulation of the mTOR signaling pathway, while inhibition of this pathway with rapamycin blocks bladder tumorigenesis in pre-clinical studies in mice. Thus, our integrated studies of mouse and human bladder cancer show that inactivation of Pten and p53 are causal events that predict poor outcome and provide a rationale for investigating mTOR inhibition as a treatment for patients with invasive bladder cancer.

In summary, our pre-clinical analyses of targeted therapies for prostate and bladder cancer using GEM models have highlighted the potential therapeutic benefit of inhibiting mTOR signaling, particularly in combination with agents targeting other signaling pathways.

170 P53 Activation as Novel Therapeutic Strategy for AML

M. Andreeff, G. Borthakur, S. Duvvuri, M. Konopleva, K. Kojima

The University of Texas M. D. Anderson Cancer Center

Development of MDM2 antagonists is currently focused on malignancies containing a wild-type p53 genotype. We have reported that the small-molecule antagonist of MDM2, Nutlin-3, strongly inhibits growth and induces p53-mediated apoptosis in hematologic malignancies. We investigated combination strategies aimed at pathologically activated pathways along with activating p53 signaling and found that p53 activation by Nutlin-3 synergizes with inhibition of Bcl-2, MEK, PI3K/mTOR, Aurora kinases, Cdk1, and FLT3. In addition, we investigated the novel HDM2 inhibitor MI63. The molecular determinants for MI63 appear similar to those for Nutlin 3a, with p53 mutation status being most important. MDM4, a transcriptional repressor of p53, has recently been reported to form a complex with p53 in mitochondria and actually enhance apoptosis. The role of MDM4 in AML is now being investigated. Another class of HDM2 antagonists has been developed that inhibits proteasome-mediated degradation of p53. JNJ-26854165 accumulates p53 and induces p53-mediated apoptosis in leukemia cell lines and primary cells. Interestingly, JNJ-26854165 delays S-phase progression and induces apoptosis independent of p53 mutation status. The exact mechanism through which the JNJ compound induces apoptosis is not known and constitutes an interesting challenge. Unexpectedly, we observed the induction of autophagy by Nutlin 3a, which was greatly enhanced by the HDAC inhibitor SAHA. The effect of autophagy on Nutlin 3a-induced apoptosis is under investigation and may constitute a defense mechanism that restricts apoptosis induction. A phase I clinical trial of Nutlin 3 in AML is moving forward and has reached at present 80 mg/m2. Serial gene expression studies of 24 p53 targets are ongoing, but a consistent induction pattern has not yet been observed. In summary, three different agents targeting the HDM2-p53 complex are under investigation. Previously established dogmas (e.g., the role of MDM4 and the need for intact p53 signaling for MDM2 inhibition) are being challenged, and new paradigms to determine the utility of HDM2-inhibition are under development. The first-in-man clinical trial of Nutlin 3a offers the opportunity to evaluate newly developed mechanistic concepts clinically.

171 Drug-Like Compounds That Inhibit HPV 16 E6

Elliot J. Androphy[1], James D. Baleja[2], and Jonathan Cherry[1]

[1]University of Massachusetts Medical School; [2]Tufts University School of Medicine

Human papillomavirus E6 proteins that cause cervical cancer bind to the ubiquitin ligase E6AP. This complex serves several functions including p53 ubiquitylation and degradation. E6AP and other E6 interacting proteins contain a common motif. We sought to discover small molecule inhibitors of E6 as an antiviral/anticancer therapy approach. Using structure-based techniques to select compounds that mimic the conserved E6 binding motif, we identified chemicals that inhibit E6 binding to E6AP and block p53 degradation. We also designed and implemented an in vitro based high-throughput screen of a chemical diversity library and isolated another set of E6 inhibitor compounds. Based on early structure-activity relationships (SAR), we have three classes of E6 inhibitors. Preliminary data demonstrate that exposure of HPV expressing cervical cancer cells to these drug-like compounds results in increased levels of p53 and its target p21cip1, whereas HPV negative cells are unaffected. One compound specifically blocked proliferation of HPV cervical cancer cells with an IC50 in the low micromolar range. Development of high activity, high potency E6 inhibitors may be useful for the pharmacologic treatment of HPV infections and malignancies.

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172 Serum Caveolin as a Marker of Src Response in Patients With Prostate Cancer Bone Metastasis

John Araujo[1], Salahldin Tahir[1], Nila Parikh[1], Geralyn Trudel[2], Gary Gallick[1], Tim Thompson[1], Christopher Logothetis[1]

[1]The University of Texas M. D. Anderson Cancer Center, Houston, Texas; [2]Bristol-Myers Squibb

Background: Therapeutic strategies using Src inhibitors are currently in clinical trials for the treatment of prostate cancer metastases in the bone. Src-family kinases (SFK) such as Src and Lyn play a key role in proliferative, invasive, bone-metastatic processes in solid tumors. Caveolin-1 (cav-1) secretion in the serum is predictive of recurrence in prostate cancer and has been identified as a critical target of Dasatinib (a SFK inhibitor) in "triple negative" breast cancer.

Methods: The Phase II clinical trial of Docetaxel (75 mg/m2 every 3 weeks) in combination with Dasatinib (100mg daily) has completed accrual. Serum has been collected to test the specific predictive markers of Src inhibition and patient response in tumor, bone, and stromal cells. We describe results of the Phase II trial and analyses by ELISA of serum Cav-1. Tissue microarrays of primary prostate cancers for Src, Lyn, and Cav-1 expression and SFK activity (pSrc) have been analyzed by IHC as well as metastatic disease from bone marrow biopsies.

Results: Results are available for the first 21 patients enrolled. Some 16/21 patients demonstrated a serum PSA decline of 50% or greater, 3/21 had a 30% decline, and 2/21 have stable PSA. Radiographically, 12/21 achieved a partial response (PR) by Recist criteria, and one of these patients achieved a complete response in bone and PR by Recist criteria in his lymph node disease. One patient was removed from study due to non-compliance, and 2 patients had progression of disease. Currently, 6 patients have stable disease.

We compared serum cav-1 at pre-treatment and 21 days post-treatment. Of the 21 patients, 18 had evaluable serum cav-1 both at baseline (prior to treatment) and at cycle 2 day 1 on treatment (day 21). Cav-1 concentrations ranged from 0.013 to 19.11 ng/ml. Radiographic outcome was assigned to these 18 patients including partial response (10/18), stable disease (6/18), and progressive disease (2/18). A trend at baseline of elevated cav-1 and subsequent decrease of less than 25% at day 21 correlated with disease less likely to respond to this combination of therapy.

Conclusions: This is the first data attempting to utilize cav-1 as a biomarker for Src inhibition therapy for the metastatic prostate cancer patient. The data strongly support examining cav-1 in detail in this setting. While this trial examines standard markers expected from any clinical trial on prostate cancer metastasis (e.g., bone turnover markers, PSA, etc.), studying markers such as cav-1 and other Src related markers is critical in planning informative trials by defining a patient population more likely to respond.

173 Inhibition of FGFR in Endometrial Cancer Cells Induces Cell Death both In Vitro and In Vivo, Demonstrating Novel Oncogene Addiction Despite Constitutive Activation of PI3K/AKT Signaling

Sara Byron[1], Michael Gartside[1], Candice Wellens[1], Mary Ann Malon[2], Feng Gao[2], Matthew Powell[2], Paul Goodfellow[2], **Pamela Pollock**[1]

[1]The Translational Genomics Research Institute, Phoenix, AZ; [2]Siteman Cancer Center, Washington University School of Medicine/Barnes-Jewish Hospital, St Louis, MO

Endometrial carcinoma is the most common gynecological malignancy in the United States. Although most women present with early disease confined to the uterus, the majority of persistent or recurrent tumors are refractory to current chemotherapies. We have identified activating mutations in FGFR2 in a total of 51/515 (10%) endometrioid endometrial cancers. Survival analyses demonstrate FGFR2 status is significantly associated with reduced progression free survival and overall survival in early stage tumors, suggesting FGFR2 status could be an independent prognostic biomarker. FGFR2 and KRAS mutations occurred in a mutually exclusive pattern, while FGFR2 mutation frequently occurs alongside PTEN mutations. Inhibition of FGFR2 by two independent shRNAs inhibited cell proliferation and induced cell death in the AN3CA cell line. Western blot analysis revealed that this induction of apoptosis following knockdown of FGFR2 correlated with inhibition of phospho-ERK and occurred in the presence of constitutively phosphorylated AKT. To verify that inhibition with a pan-FGFR inhibitor was a viable therapeutic option in this tumor type, a panel of endometrial cell lines was treated with the pan-FGFR inhibitor PD173074 (Calbiochem). The three cell lines with mutant FGFR2 (AN3CA, MFE296, MFE280) were 10-40x more sensitive to inhibition with PD173074. Notably the most sensitive line has loss-of-function mutations on both PTEN alleles, and Western blot data confirmed this cell death occurred in the presence of constitutive AKT signaling. These data suggest that endometrial cancer cells with activated FGFR2 demonstrate a novel form of oncogene addiction. FGFR inhibition with PD173074 significantly inhibited tumor growth in the AN3CA human endometrial tumor xenograft model (activated FGFR2) but not in the HEC1A xenograft model (FGFR2 wildtype). Six of eight AN3CA tumors showed regression with a mean tumor shrinkage of 51%, and the remaining two tumors demonstrated tumor growth inhibition of 91% compared to the mean tumor weight of the control group ($P<0.001$). Ongoing basic studies are concentrating on elucidating the novel caspase-independent, mitochondrial-dependent, CHX-dependent mechanism of cell death in response to FGFR inhibition with PD173074, and ongoing translational studies involve validating several multi-target kinase inhibitors including TKI258 and Brivanib as clinically relevant FGFR inhibitors in endometrial cancer.

174 Targeting Developmental Pathways Such as Wnt/Frizzled Signaling in Cancer

Minyong Chen, Jiangbo Wang, Jiuyi Lu, Michael C. Bond, Xiu-Rong Ren, H. Kim Lyerly, Larry S. Barak, **Wei Chen**

Department of Medicine, Duke University Medical Center, Durham, NC

The Wnt signaling pathway plays a fundamental role in the developing embryo by directing tissue patterning and in the mature organism by maintaining tissue homeostasis. Upregulation of Wnt-ligand/Frizzled-receptor signaling and other developmental pathways play a key role in the pathogenesis of many devastating malignancies. Frizzled receptors are expressed at cell surface, rendering them susceptible to antibodies and small molecule inhibitors. Currently, there exist neither drug candidates nor tool compounds that modulate Wnt-mediated Frizzled receptor trafficking and subsequent Wnt signaling. We examined libraries of FDA-approved drugs for their utility as Frizzled internalization modulators, employing a primary cell-based, high throughput GFP-fluorescence assay that imaged Frizzled1 endocytosis. We now report that a FDA-approved and marketed drug used for the treatment of none-Wnt related diseases promotes Frizzled1 endocytosis but inhibits Wnt3A-stimulated ß-catenin stabilization and LEF/TCF reporter activity. Additionally, following drug-induced internalization, the Frizzled1 receptor co-localizes in vesicles containing Transferrin and agonist-activated ß2-adrenergic receptor. Therefore, this FDA-approved drug may serve as a negative modulator of Wnt/Frizzled1 signaling by depleting the membrane of receptors and may provide a valuable means to study the physiological consequences of Wnt signaling. Moreover, our data suggest a potential new therapy that may be effective in cancers of colon, breast, lung, and liver.

175 Smart Bombs for Cancer: Translating Theory Into Therapy

Samuel R Denmeade[1], Soren B. Christensen[2], **John T. Isaacs**[1]

[1]The Sidney Kimmel Comprehensive Cancer Center, Departments of Oncology and Urology and The Brady Urological Institute, The Johns Hopkins University School of Medicine, Baltimore, MD; [2]Department of Medicinal Chemistry, University of Copenhagen, Denmark

We used structure-activity relationships and chemistry of a plant derived lipolipic sesquiterpene lactone, Thapsigargin(T), to develop analogs that retain high cell penetrance and potent (nM) inhibition of intracellular sarco-/endoplasmtic calcium pumps. Due to these abilities, T analogs concentrate within cells disrupting intracellular calcium metabolism, killing cells irrespective of phase of the cell cycle. Lack of selectivity prevents use of these T analogs as systemic drugs.

To restrict the toxicity of T analogs to prostate cancer cells, they were conjugated to peptides whose sequence was designed so that they are efficient substrates only for a prostate specific protease (PSA, hK2, and PSMA), characteristically express extracellular by prostate cancer cells and not blood or non-prostate tissue proteases. These peptide conjugations produce prodrug "smart bombs" that are deliverable via the circulation to metastatic sites without excessive host toxicity since only in the extracellular fluid in sites of cancer are prodrug smart bombs enzymatically hydrolyzed by prostate-specific proteases to produce sufficient amounts of killing analog to sterilize the cancer area. Besides targeting toxicity, this prodrug smart bomb design has two major advantages over previous targeting attempts with antibodies. First, due to the nature of the enzymatic activation, there is tremendous amplification of stoichiometry of liberated analog per enzyme molecule. Second, since this activation occurs extracellularly, once formed the lipophilic analog does not re-enter general circulation restricting its toxicity, but instead it concentrates in all cells in its vicinity regardless of whether they express activating enzyme.Thus, a strong "by-stander effect" is produced, solving a major problem of tumor cell heterogeneity that limits therapies requiring expression by all cancer cells.

These prodrug smart bombs have undergone pre-clinical xenograft and host toxicity evaluation. Based upon these results, GenSpera Inc completed GMP production and animal toxicity profiling of a PSMA activated Thapsigargin prodrug smart bomb, termed G-202. An IND has been filed with the FDA for G-202, and Phase 1/II trials are planned to start in late 2009.

176 Human Monoclonal Antibodies and Engineered Antibody Domains Targeting Cancer-Related Proteins

Yang Feng[1], Xiaodong Xiao[1], Zhongyu Zhu[1,2], Weizao Chen[1], Emily Streaker[1], Mitchell Ho[3], Ira Pastan[3], **Dimiter S. Dimitrov**[1]

[1]Protein Interactions Group, CCRNP, National Cancer Institute (NCI), National Institutes of Health (NIH), Frederick, MD; [2]BRP, SAIC-Frederick, Inc., NCI, Frederick, MD; [3]Laboratory of Molecular Biology, Center for Cancer Research, NCI, NIH, Bethesda, MD

We have identified several human monoclonal antibodies (hmAbs) against components of the IGF system, including IGF-II, IGF-I, and BP2, as well as against DR4, DR5, mesothelin, and CD22 by using our phage-displayed libraries. One of these antibodies, m610, which recognizes both the mature and the premature forms of IGF-II, inhibited the proliferation of the neuroblastoma cell lines tested. Because IGF-II secretion is upregulated in many types of tumors, especially childhood malignancies such as neuroblastoma, osteosarcoma, and Ewing's sarcoma, therapy targeting ligands could be beneficial. This antibody is also able to deplete soluble IGF-II from serum and tissues, which blocks autocrine and paracrine mechanisms. It not only blocks signals through IGF-IR, but also blocks IGF-II binding to the insuling receptor (IR) and activation of IR. Another antibody, m708, is cross-reactive for both IGF-I and IGF-II. These antibodies, while already with nM affinity, were further affinity matured. We have also identified and characterized to various extents antibodies against mesothelin, CD22 , and DR4 and DR5. In an attempt to increase penetration into solid tumors and epitopes that are not accessible for full-size antibodies, we constructed a large (size about 2.5 · 1010) domain antibody (dAb) library by grafting human antibody heavy chain complementarity determining regions (CDRs) 2 and 3 (H2s, H3s) into their cognate positions in a human heavy chain variable domain (VH) scaffold and mutagenizing the CDR1 (H1). We also constructed a novel type of dAb library containing CDRs in non-cognate positions based on our previous library where H1 was replaced by a library of human light chain CDR3s (L3s), thus combining three most diversified fragments (L3, H3, and H2) in one VH scaffold. Three novel high-affinity dAbs against the human insulin-like growth factor 2 (IGF-2) were selected from the new library including one cross-reactive with IGF-1 but only one dAb with relatively modest affinity was found by panning of the previously constructed library. The newly identified dAbs were highly soluble, expressible, and stable and may have potential as candidate cancer therapeutics. These libraries could be used for selection of a variety of other dAbs with potential utility as anti-cancer agents.

177 Mutant PIK3CA Activates Multiple Oncogenic Pathways

John Gustin, Bedri Karakas, Michaela Higgins, Marc Rosen, Samuel Denmeade, Antonio Wolff, **Josh Lauring**, Ben Ho Park

Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins

The phosphatidylinositol 3-kinase subunit PIK3CA is frequently mutated in human cancers. Here we used gene targeting to "knock in" PIK3CA mutations into human breast epithelial cells to identify new therapeutic targets associated with oncogenic PIK3CA. Mutant PIK3CA knockin cells were capable of epidermal growth factor and mTOR-independent cell proliferation that was associated with AKT, ERK, and GSK3beta phosphorylation. Paradoxically, the GSK3beta inhibitors lithium chloride and SB216763 selectively decreased the proliferation of human breast and colorectal cancer cell lines with oncogenic PIK3CA mutations and led to a decrease in the GSK3beta target gene CYCLIN D1. Oral treatment with lithium preferentially inhibited the growth of nude mouse xenografts of HCT-116 colon cancer cells with mutant PIK3CA compared with isogenic HCT-116 knockout cells containing only wild-type PIK3CA. Our findings suggest GSK3beta is an important effector of mutant PIK3CA, and that lithium, an FDA-approved therapy for bipolar disorders, has selective antineoplastic properties against cancers that harbor these mutations.

178 Tamoxifen and Letrozole Resistance Is Reversed In Vitro and In Vivo by the IGF-1R/Insr Inhibitor, BMS-754807

Paul Haluska[1], Xiaonan Hou[1], Fei Huang[2], Sean Harrington[1], Ann Greer[2], Luciana Macedo[3], Angela Brodie[3], Dean B. Evans[4], Joan Carboni[2], and Marco Gottardis[2]

[1]Mayo Clinic, Rochester, MN; [2]Bristol-Myers Squibb Co., Princeton, NJ; [3]University of Maryland, Baltimore, MD; [4]Novartis Pharma AG, Basel, Switzerland

Resistance to hormonal therapy is a clinically unmet need in breast cancer. IGF signaling has been identified as a major mechanism of resistance to hormonal therapy. As components of the IGF signaling pathway are expressed in most breast cancers, the development of IGF-1R monoclonal antibody (mAb) and tyrosine kinase inhibitors (TKI) are active areas of clinical investigations. Key distinctions between the mAb and TKIs are the differences in insulin receptor (InsR) inhibition. While targeting the InsR with TKIs may have a theoretical liability of hyperglycemia, targeting only the IGF-1R may have the theoretical liability of incompletely blocking IGF signaling. As InsR isoform A expression, which can transduce IGF-II-mediated proliferation, is higher in breast cancers compared to normal breast tissue, we investigated whether IGF-1R or IGF-1R/InsR inhibition was sufficient for overcoming resistance to hormonal therapy. For these studies, we used either hormone therapy-naïve or -resistant variants of the breast cancer model, MCF-7/AC-1, which has been engineered to stably express human aromatase. We employed and compared a novel, potent dual kinase inhibitor of IGF-1R/InsR, BMS-754807, which is currently in early clinical investigations, with the IGF-1R mAb 391. In vitro, BMS-754807 demonstrated profound synergy in combination with tamoxifen and letrozole (median effect CI values <0.1). In vivo, BMS-754807 enhanced the anti-tumor activity of tamoxifen and letrozole in hormone-naïve tumors and induced regression of tumors resistant to tamoxifen or letrozole when combined with letrozole. This activity was not observed with mAb therapy, which resulted in greater upregulation of InsR-A and erbB receptor expression and activation, suggesting greater susceptibility to resistance pathways. Gene expression profiling experiments to compare the difference between the effects of tamoxifen in combination with BMS-754807 and with mAb revealed alternative pathway signaling is one of the potential mechanisms of resistance.

In summary, combined hormonal therapy with BMS-754807 overcomes primary and secondary resistance to tamoxifen and letrozole and was well tolerated. IGF-1R blockade with a mAb alone is insufficient to overcome resistance and induces InsR overexpression. IGF signaling through either InsR or IGF-1R may be a major mechanism of resistance to hormonal therapy. These data suggest that blockade of IGF-1 and IGF-II from activation of IGF-1R and InsR, with agents such as BMS-754807, have promise in extending the benefits of hormonal therapy in breast cancer.

179 Cdk1 Inhibition Disrupts BRCA1-Dependent S Phase Checkpoint Control and Selectively Sensitizes Cancer Cells to DNA Damaging Treatments

Neil Johnson[1], Dongpo Cai[1], Richard D. Kennedy[1], Shailja Pathania[1], Mansi Arora[2], Yu-Chen Li[1], Alan D. D'Andrea[1], Jeffrey D. Parvin[2], **Geoffrey I. Shapiro**[1]

[1]Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA; [2]Ohio State University, Columbus, OH

Cdk2 and cdk1 are individually dispensable for cell cycle progression in cancer cell lines because they are able to compensate for one another. However, we have demonstrated that shRNA-mediated depletion of cdk1 alone or small molecule cdk1 inhibition abrogates S phase cell cycle arrest in NCI-H1299 and A549 non-small cell lung cancer cells after exposure to a range of DNA damaging treatments, including ionizing radiation and cisplatin. In the absence of cdk1 activity, cell cycle progression continued after DNA damage, with death of cells from the late S and G2 phases. In contrast, cdk2 depletion did not affect DNA damage-induced S phase checkpoint control. Following ionizing radiation in the absence of cdk1, activation of ATM was intact, as was DNA end resection (measured by the formation of RPA32 foci at sites of DNA damage) and the activation of ATR. Whereas phosphorylation of chromatin-bound ATM/ATR substrates such as H2AX and Rad17 occurred normally, the phosphorylation of non-chromatin bound substrates, including Chk1 and Chk2, was compromised, leading to the failure of checkpoint pathway activation and the persistent activity of novel cyclin B-cdk2 complexes in cdk1-depleted cells. BRCA1 is a key mediator of ATR/ATM responses to DNA damage and facilitates ATR/ATM phosphorylation of a number of their non-chromatin-bound substrates. We have shown that after DNA damage, in the absence of cdk1, BRCA1 is not recruited to sites of damaged DNA. Furthermore, cdk1 directly phosphorylates BRCA1 at S1189/S1191 and S1497, and mutation of these sites substantially reduces the ability BRCA1 to form foci at sites of DNA damage. Cdk1 knockdown results were confirmed using the selective small molecule inhibitor RO-3306. Abrogation of checkpoint control after cdk1 depletion or inhibition in non-small cell lung cancer cells sensitized them to DNA damaging agents, measured by long-term colony formation assays. Combined depletion of cdk1 and BRCA1 did not sensitize cells to DNA damage better than depletion of either alone, confirming that cdk1 and BRCA1 operate in the same pathway during the DNA damage response. Additionally, in non-transformed retinal pigment epithelial cells, reduced cdk1 activity caused more potent G2/M arrest than in transformed cell, and antagonized the response to subsequent DNA damage. Cdk1 inhibition may therefore selectively sensitize BRCA1 proficient cancer cells to DNA damaging treatments by disrupting BRCA1 function.

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180 A Novel Strategy for Targeting Wnt Signaling in Advanced Metastatic Colorectal Cancer

Kimberly Jones[1,2], Reid Phelps[2,3], Sunil Sharma[1,2], **David A. Jones**[2,3]

[1]Department of Medicine, [2]Huntsman Cancer Institute, and [3]Department of Oncological Sciences, University of Utah, Salt Lake City, UT

Initiation and progression of colorectal cancer is thought to occur as a result of sequential mutations in the APC tumor suppressor gene followed by mutations in KRAS or p53. There are limited treatment options for patients with metastatic colorectal cancers that harbor mutated KRAS given that anti-EGFR therapies are ineffective in this patient population. Additionally, approximately 30% of patients with KRAS wild-type tumors fail to respond to anti-EGFR therapies, likely due to downstream mechanisms of resistance. Recent work in our group has revealed an expected connection between aberrant Wnt signaling, cyclooxygenase-2 activity and the activation of KRAS in directing intestinal cell proliferation. Using zebrafish genetics in juxtaposition with human tumor specimens and human cell lines, we have shown that nuclear accumulation of ß-catenin and consequential intestinal cell proliferation requires the activity of both COX-2 and KRAS. Mechanistically, KRAS promotes the nuclear localization of stabilized ß-catenin through activation of RAF1 but independently of the MEK1 signaling pathway. This hypothesis was further supported by knockdown experiments in SW-480 APC mutated cell line with oncogenic RAS, where siRNA knockdown of RAF1 restored localization of ß-catenin to the cytoplasm but depletion of MEK1 did not. The blockage of COX-2 (inhibitor NS-398) also resulted in the exit of ß-catenin from the nucleus in comparison to control, untreated cells. We also examined 20 matched sets of human tissues that were grossly uninvolved, FAP adenomas, and sporadic carcinomas. We found an increase of ß-catenin in the adenomatous tissues, but it appeared largely cytoplasmic. In contrast, nuclear ß-catenin was readily detected in carcinomas (Cell. 2009 May 15:137(4):623-34). Based on this new mechanistic insight, we hypothesize that combining an anti-RAF therapy with a COX-2 inhibitor will block the nuclear localization of ß-catenin and Wnt signaling via a novel mechanism. In a Phase I study, we plan to use this combination in conjunction with biomarker analysis on tumor tissue to measure for the expected effect of decreased production of ß-catenin, decreased nuclear staining of ß-catenin. These measurements will be compared to pretreatment specimens if available or historical controls.

181 Wnt/ß-Catenin Pathway Activation Is Specific to Basal-Like Breast Cancers and Predicts Poor Outcome

Andrey I. Khramtsov, Galina F. Khramtsova, Maria Tretiakova, Dezheng Huo, Olufunmilayo I. Olopade, **Kathleen H. Goss**
University of Chicago, Chicago, IL

While Wnt/ß-catenin pathway activation has been strongly implicated in mouse models of breast cancer, there is contradictory evidence for its importance in human sporadic breast cancer. To explore its role in discrete breast cancer subtypes, immunohistochemical analysis for ß-catenin, the major effector of the pathway, was performed on breast cancer tissue microarrays that contained luminal A, luminal B, HER2-positive, and basal-like invasive cancers as well as ductal carcinoma in situ (DCIS) lesions. We found that nuclear and cytosolic accumulation of ß-catenin, a readout of Wnt/ß-catenin pathway activation and ß-catenin transcriptional activity, was specifically associated with those breast cancers with a basal-like phenotype. In contrast, membrane associated ß-catenin was observed in all breast-cancer subtypes, and its expression decreased with tumor progression. Moreover, nuclear and cytosolic ß-catenin were associated with other markers of the basal-like phenotype, including nuclear hormone receptors and HER2 negativity, cytokeratin 5/6 and vimentin expression, and enrichment of stem cells. Importantly, this subcellular localization of ß-catenin was associated with a poor outcome and is more frequent in tumors from African American patients. In addition, ß-catenin accumulation appears to be an early event in basal-like invasive tumors since it was found exclusively in basal-like DCIS lesions but not other in situ carcinomas subtypes. Collectively, these data suggest that Wnt/ß-catenin activation may be an important feature of basal-like breast cancers, one that might be useful to target therapeutically in this aggressive breast cancer subtype. We are currently conducting early translational and preclinical studies to test the efficacy of Wnt/ß-catenin pathway antagonists specifically in basal-like breast cancer models.

182 Development of Therapeutic Antibodies Targeting Notch3 in Lung Cancer

Luping Lin, Raymond Mernaugh, Robert Carnahan, David P. Carbone, **Thao P. Dang**
Vanderbilt University Medical Center

The Notch receptors are essential for both normal development and tumor growth in many human cancers. Notch3 is expressed in 40% of all lung cancers. Inhibiting this pathway results in reduced tumor growth in lung in vitro as well as in vivo. Thus, this pathway represents a potentially important target for therapeutic development. Here we report the early results of a strategy to inhibiting Notch3 signaling through the development of Notch3 monoclonal antibodies.

Using a Notch3 peptide library, we discovered two regions of the extracellular domain, believed to be the binding sites for the Notch3 ligand, Jagged1. These regions were cloned, and the resulting recombinant proteins were used to immunize and boost mice in vivo. We demonstrated that antisera from these mice immunized with portions of the receptor extracellular domain can inhibit Notch3 activation. Following fusion of spleen cells with myeloma cells, the hybridoma clones were screened with ELISA and specific antibody production. Six hybridomas were selected and subcloned according to immunoblotting, immunoprecipitation, and the ability to inhibit Notch3 cleavage. These candidate clones were found to specifically inhibit Notch3 activation but not Notch1. Further testing is ongoing to validate these findings as well as to determine affinity and anti-tumor activity.

In summary, our data indicated that epitope-mapping using small peptides can allow the development of monoclonal antibody to specifically inhibit Notch3 signaling and serve as a strategy for the development of future therapeutics for patients with lung cancer.

183 SCH727965, A Novel Potent Cyclin Dependent Kinase Inhibitor, Inhibits Pancreatic Tumor Growth and Ras-Mediated Tumorigenic Signaling

Anjali Mishra[1], Georg Feldmann[2], Ignacio Garrido-Laguna[1], Christopher J. Strock[1], Rajat Bannerji[3], Manuel Hidalgo[1], Anirban Maitra[1,2], **Barry D. Nelkin**[1]

Departments of [1]Oncology, [2]Pathology, Johns Hopkins University School of Medicine, Baltimore, MD; [3]Schering-Plough Research Institute, Kenilworth, NJ

Cyclin-dependent kinase 5 (CDK5), a kinase associated with neuronal migration, is active in human pancreatic cancer. Abrogation of CDK5 function using a dominant-negative protein, RNA interference, or pharmacological disruption significantly mitigated invasion, migration, and anchorage independent growth in vitro and orthotopic tumor formation and systemic metastases in vivo. CDK5 blockade resulted in inhibition of RalA, a key effector pathway downstream of oncogenic Ras, central to tumorigenesis in pancreatic cancer. Restitution of Ral function rescued the effects of CDK5 inhibition in pancreatic cancer cells. Thus, CDK5 is important for Ras signaling in pancreatic cancer, suggesting that CDK5 inhibition may be an effective therapeutic strategy in pancreatic cancer. To begin to explore the therapeutic potential of CDK5 inhibitors in pancreatic cancer, we have examined the effect of the semi-selective CDK5 inhibitor SCH727965 (Schering-Plough). SCH727965 is a potent inhibitor of CDK1, CDK2, CDK5 and CDK9, with in vitro IC50 values of 3, 1, 1 and 4 nM, respectively. In this study, we evaluated the effect of SCH727965 on pancreatic tumor growth and migration. MTT assay results demonstrated that the IC50 for 2 pancreatic tumor cell lines, MiaPaCa-2 and Panc1.98, was 12nM and 24nM respectively. Migration and anchorage independent growth were significantly more sensitive to SCH727965. Thus, Boyden chamber assay and soft agar assays indicated 50% inhibition of migration and soft agar growth inhibition at 5nM, and complete inhibition at 10nM drug concentration—concentrations at which the effects on anchorage dependent growth were relatively minor. Tumors from low passage pancreatic tumor xenografts, or from pancreatic cell line orthotopic xenografts, showed 40–75% reduction of tumor growth after treatment with SCH727965, with significant reduction of metastases. SCH727965 treatment resulted in inhibition of RalA activation. Ral activity could be restored by enforced RalA activation, via expression of Rgl2-CAAX, a constitutively active form of a RalGEF (Ral guanine exchange factor) gene. Our results show that SCH727965 is very effective against pancreatic cancer growth and metastasis. These results also suggest that SCH727965 blocks pancreatic tumor growth, migration and metastasis, at least in part by inhibition of dysregulated Ras signaling, by inhibiting CDK5. (R01 CA134767.)

184 Targeting Chk1 With AZD7762 to Improve Gemcitabine-Radiation Efficacy in Pancreatic Cancer

Meredith A. Morgan[1], Leslie A. Parsels[2], Joshua D. Parsels[2], Mary A. Davis[1], Linda Hylander-Gans[4], Sonya D. Zabludoff[4], Diane M. Simeone[3], Jonathan Maybaum[2], Theodore S. Lawrence[1]

Departments of [1]Radiation Oncology, [2]Pharmacology, and [3]Surgery, The University of Michigan Medical School, Ann Arbor, MI; [4]AstraZeneca R&D Boston, Waltham, MA

The combination of gemcitabine and radiation is now a standard therapy for locally advanced pancreatic cancer and was recently demonstrated to be statistically superior to gemcitabine alone. Our goal is to improve therapy for pancreatic cancer by the addition of molecularly targeted agents to gemcitabine-radiation. Our previous work showed that Checkpoint kinase 1 (Chk1) inhibition enhances sensitivity to gemcitabine as well as radiation. Therefore, in the present study we investigated the novel Chk1/2 inhibitor, AZD7762 (AstraZeneca) currently in Phase I clinical trial, in combination with gemcitabine-radiation in both in vitro and in vivo pancreatic cancer models. We hypothesized that AZD7762 would sensitize pancreatic cancer cells/xenografts to gemcitabine and radiation. We tested this hypothesis in vitro by measuring the clonogenic survival of MiaPaca2 cells in response to AZD7762, gemcitabine, and radiation. Furthermore, the ability of AZD7762 to sensitize to gemcitabine and radiation in vivo was tested in MiaPaca2- and patient-derived tumor xenografts. We found that AZD7762 produced significant radiosensitization (RER 1.5 ± 0.1) and further enhanced gemcitabine-mediated radiosensitization (RER Gem 1.2 ± 0.1 vs. Gem + AZD7762 1.9 ± 0.2, $P<0.05$) in MiaPaca2 cells. In MiaPaca2-derived tumor xenografts, growth was significantly inhibited by AZD7762 in response to gemcitabine-radiation (median tumor volume doubling time Gem + RT 75 vs. Gem + RT + AZD7762 >109 days, $P<0.05$). AZD7762 treatment was associated with inhibition of Chk1 activity, evidenced by decreased pChk1(296) as well as increased DNA damage, evidenced by increased pChk1(345) and γH2AX. In patient-derived pancreatic tumor xenografts treated with gemcitabine, radiation, and/or AZD7762 the time until tumor volume doubling was prolonged by AZD7762 in response to radiation or gemcitabine-radiation (RT 32, AZD7762 + RT 45, Gem + RT 49, Gem + RT + AZD7762 59 days). We conclude that Chk inhibition by AZD7762 induces sensitization to radiation as well as gemcitabine-radiation in pancreatic cancer cells and tumors. These data support our current goal of combining AZD7762 with gemcitabine-radiation clinically in patients with pancreatic carcinoma.

185 Pituitary Adenylyl Cyclase Activating Peptide (PACAP) Regulates Medulloblastoma Pathogenesis in ptc1 Mutant Mice and Counter-Regulates Multiple Hedgehog Target Genes

Pawel Niewiadomski, Joseph Cohen, Jennifer Kong, Daniel Resnick, Annie Zhujiang, Linda M. Liau, **James A. Waschek**

University of California, Los Angeles

In flies and vertebrates, Hedgehog proteins and cAMP-dependent protein kinase A (PKA) generally play opposing roles in developmental patterning events. Humans and mice heterozygous for mutations in the Sonic hedgehog (SHH) receptor gene patched-1 (ptc1) have an increased incidence of certain types of cancer, including medulloblastoma (MB), a highly aggressive tumor of young adults and children. Receptors for pituitary adenylyl cyclase activating peptide (PACAP) and SHH are coexpressed in the germinal centers that are thought to give rise to MB. We hypothesized that loss of PACAP might synergize with the ptc1 mutation to increase susceptibility to these tumors. Mutation of a single copy of PACAP increased MB incidence approximate 2.5-fold, to 66%. Moreover, tumors in ptc1+/- mice could be detected in vivo by positron-emission tomography (PET) at least 6 weeks before the onset of symptoms. To identify novel PACAP-sensitive target genes that might be involved in MB, cultures of mouse cerebellar granule precursor, the cells thought to give rise to MB, were treated with SHH, PACAP, or the combination. The expression of the great majority of genes induced by SHH were blocked by PACAP, including several novel SHH target genes. These genes were found to be expressed in the cerebellum at highest levels during postnatal development and were also expressed in mouse MB tumors. The results (1) implicate PACAP as a physiological PKA activating factor that regulates the incidence and/or growth of hedgehog pathway-associated medulloblastoma tumors in mice and (2) identify a set of novel genes that are cross-regulated by these signaling pathways.

186 MDA-7 Kills RCCs Via Ceramide/CD95 and ER Stress Signaling

Margaret A. Park[1], David T. Curiel[5], Christina Voelkel-Johnson[6], Steven Grant[1,2,3,4], Paul B. Fisher[3,4], **Paul Dent**[1,3,4]

Departments of [1]Biochemistry, [2]Medicine, [3]Human and Molecular Genetics, [4]Institute of Molecular Medicine, Virginia Commonwealth University, Richmond, VA; [5]Division of Human Gene Therapy, Department of Medicine, and the Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL; [6]Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC

Melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24) is a novel cytokine displaying selective apoptosis-inducing activity in transformed cells without harming normal cells. The present studies focused on clarifying the mechanism(s) by which a GST-MDA-7 fusion cell survival of human renal carcinoma cells (RCCs) in vitro. GST-MDA-7 caused plasma membrane clustering of CD95 and formation of a DISC containing FADD and pro-caspase 8. GST-MDA-7 lethality was suppressed by inhibition of caspase 8 or by over-expression of c-FLIP-s, but only weakly by inhibition of cathepsin proteases. GST-MDA-7-induced CD95 clustering and apoptosis was blocked by knockdown of acidic sphingomyelinase or to a greater extent ceramide synthase 6 expression. GST-MDA-7 killing in RCCs was in parallel dependent on inactivation of ERK1/2 and on CD95-induced p38 MAPK and JNK1/2 signaling. Knockdown of CD95 expression abolished GST-MDA-7-induced phosphorylation of protein kinase R-like endoplasmic reticulum kinase (PERK) and eIF2 alpha. GST-MDA-7 lethality was suppressed by knock-out or expression of a dominant negative PERK that correlated with reduced JNK1/2 and p38 MAPK signaling and maintained ERK1/2 phosphorylation. GST-MDA-7 caused vacuolization of LC3-GFP endosomes through a mechanism that was CD95-dependent and whose formation was suppressed by knockdown of ATG5 expression. GST-MDA-7 enhanced expression of ATG5 in a PERK-dependent fashion and knockdown of ATG5 suppressed GST-MDA-7-toxicity. Our data demonstrate that, in kidney cancer cells, GST-MDA-7 induces ceramide-dependent activation of CD95, which is causal in promoting an ER stress response that activates multiple pro-apoptotic pathways to decrease renal cancer cell survival.

187 Investigating the Mechanisms Determining Sensitivity or Resistance to SMAC-Mimetic JP1201 in Lung Cancer Cell Lines

Sean Peterson, Rachel M. Greer, Michael Peyton, John Minna, Xiaodong Wang

University of Texas Southwestern Medical Center at Dallas

Many cancers are resistant to apoptotic signals, making them relatively resistant to standard anticancer chemotherapies. Thus, an attractive avenue for sensitizing such cancers to existing drugs is to attack the mechanism of resistance to apoptosis. One source of this resistance is upregulation of Inhibitors of Apoptosis Proteins (IAPs), which blocks the activity of caspases. Normally, when the intrinsic pathway of apoptosis is activated, the second mitochondrial activator of caspases (SMAC) is released from the mitochondria and antagonizes IAPs by binding to them. Increased expression of IAPs compensates for SMAC activity and represses apoptosis. To investigate the therapeutic potential of sensitizing lung cancers to apoptosis, members of the P01CA095471 team synthesized a compound that mimics the activity of SMAC and sensitized cancer cell lines to apoptotic stimuli (Li et al. 2004). In collaboration with the Lung Cancer SPORE (P50CA70907) team, subsequent survey of 50 human non-small cell lung cancer (NSCLC) lines revealed that ~25% were sensitive to the SMAC-mimetic alone, indicating that they generated an apoptotic signal inhibited by IAPs. This apoptotic signal was found to be autocrine secretion of TNF, providing a potential biomarker predictive of SMAC-mimetic sensitivity. In response to TFN& signaling, the SMAC-mimetic promotes the activation of caspase 8 through a complex dependent on RIPK1 (Petersen et al. 2007). However, the majority of NSCLC cell lines did not respond to the SMAC-mimetic, even in combination with TNF. We have found that SMAC-mimetic induces the degradation of cIAP1 and cIAP2, with the degradation of cIAP2 requiring cIAP1. Some cells upregulate cIAP2 transcription in response to TNF and the return of cIAP2 blocks formation of the RIPK1-caspase 8 complex. Another class of cells is unable to respond to TNF. To sensitize resistant cells to the SMAC-mimetic, we targeted parallel pathways that regulate cIAP2. Inhibiting AKT or the EGFR proved effective in sensitizing cells to SMAC-mimetic. By contrast, when SMAC-mimetic was combined with standard chemotherapy agents in tests of a panel of SMAC-mimetic "resistant" NSCLC tumor lines, we found in every line at least one example of dramatic synergy (IC50 decreases of 20 to 10,000 fold) with SMAC-mimetic. In addition, there was marked heterogeneity between NSCLCs, with tumors being killed by different SMAC-mimetic + chemotherapy combinations. While no sensitization was seen with cisplatin, there were multiple examples of sensitization to paclitaxel or vinorelbine. This provides a clear path for clinical testing of SMAC-mimetic in NSCLC patients.

188 Targeting Aurora Kinase in Aggressive B-Cell Non-Hodgkin's Lymphomas

Wenqing Qi, Laurence Cooke, Xiaobing Liu, Daniel Persky, Thomas P. Miller, **Daruka Mahadevan**

Arizona Cancer Center, Tuscon, AZ

Aurora kinases (A and B) are oncogenic serine/threonine kinases that play central roles in the mitotic phase of the cell cycle. Over-expression and aberrant activation of Aurora over-rides mitotic and spindle check points leading to aneuploidy and make Auroras attractive therapeutic targets. We hypothesized that Auroras are over-expressed in aggressive B-cell NHL, an Aurora ATP-site small molecule inhibitor is effective in promoting apoptosis in cell culture and tumor growth inhibition (TGI) in mouse xenograft model(s) of NHL, and that an Aurora SMI will be safe and effective in treating patients with relapsed aggressive B-cell NHL in early phase clinical trials. In order to analyze Aurora expression, tissue microarrays of 25 cases of DLBCL and MCL were constructed and are being evaluated by IHC. In one patient with MCL (spleen), intense staining (3+) for Aurora A (nucleus and cytoplasm) and Aurora B (nucleus) was demonstrated. We analyzed the LLMPP database for Aurora A and B expressers in MCL and showed a worse survival in those with over-expression ($p<0.01$). Western blotting analysis of 13 B-cell NHL cell lines (MCL, DLBCL and TFL) for Aurora showed significant over-expression compared to normal B-cells. Aurora A knockdown by shRNA in the B-NHL cell lines showed inhibition of mitosis with a polyploid phenotype (4n, 8n) that ends in apoptosis. The Aurora A specific inhibitor (MLN8273) phenocopies shRNA knockdown with associated inhibition of proliferation (IC50=0.05 microM) and promotion of apoptosis in a dose-dependent manner. Combination of MLN8273 with a microtubule targeting agent (taxol) to abrogate the spindle checkpoint is synergistic. Two mouse MCL (Granta 519) xenograft models are underway evaluating efficacy (TGI), safety, and survival of MLN8273 alone and in combination with taxotere or rituximab respectively. A phase II study with MLN8273 for relapsed/refractory NHL is underway, with plans to investigate Aurora A inhibition on apoptosis, cytokine profiling (pre- and post- treatment), and pre-and post- treatment core biopsies for Aurora expression and biomarkers of inhibition. The data suggest inhibition of Auroras offers a promising treatment strategy for patients with aggressive B-cell NHL.

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189 RNAi-Mediated Selective Inhibition of PI3K/Akt Pathway Components

Piotr G. Rychahou, Pat Gulhati, **B. Mark Evers**

Markey Cancer Center, University of Kentucky, Lexington KY

Colorectal cancer (CRC) is the second-leading cause of cancer deaths in the United States. When localized to the mucosa and sub-mucosa of the bowel wall (Stage I), the 5-year survival approaches 100%; however, metastasis to the lymph nodes (Stage III) results in a precipitous decrease in 5-year survival, and systemic metastasis to the liver (i.e., Stage IV) is associated with a 5-year survival of less than 5%. Activation of phosphatidylinositol-3-kinase (PI3K), a ubiquitous lipid kinase composed of an 85kDa regulatory subunit (p85) and a 110kDa catalytic subunit (p110), and its downstream effector protein, Akt, is associated with the growth and progression of a number of cancers, including CRC. We have shown altered PI3K/Akt expression in CRCs and surrounding stroma with increased expression of the p85α subunit and Akt2 in CRCs of increasing stage. Targeted inhibition of either p85α or p110α by RNA interference (RNAi) increases sensitivity of resistant CRCs to the effects of chemotherapy and significantly suppresses CRC metastasis to the liver in pre-clinical animal models. In recent findings, we have shown that suppression of Akt2 expression in highly metastatic CRC cells inhibits their ability to metastasize (PNAS, 2008). Overexpression of wild-type Akt1 did not restore metastatic potential in cells with down-regulated Akt2, thus suggesting non-redundant roles for the individual Akt isoforms. The central hypothesis of this project is that CRC growth and progression are augmented by increased p85α and Akt2 expression; the selective inhibition of PI3K/Akt components can suppress CRC growth and metastasis and, furthermore, can sensitize resistant CRCs to chemotherapeutic agents. The long-term goal of this project is to develop more selective therapies for CRC progression and metastasis based upon selective RNAi to PI3K pathway components. The safety of RNAi in clinical studies has been documented by recent clinical trials. Therefore, we suspect that either selective Akt inhibitors or RNAi directed to specific components will result in a safe and effective alternative for chemotherapy. Newer and longer-acting RNAi has now been developed by a number of companies. A major obstacle remains drug-delivery techniques that can provide more specific uptake into cancer cells. The ability to selectively inhibit certain components of the PI3K pathway represents an exciting option in humans, since animal studies have now demonstrated feasibility and safety. Our studies have identified fundamental and functional differences in PI3K isoforms that represent novel targets for drug design and delivery.

190 Targeting Notch Signaling in Ovarian Cancer

Alexander Stoeck, Joon T. Park, Xu Chen,Tian-Li Wang, Le-Ming Shih

Departments of Pathology, Oncology, and Gynecology and Obstetrics, Johns Hopkins Medical Institutions Baltimore, MD

Ovarian serous carcinoma represents one of the most aggressive neoplastic diseases with a high rate of recurrence and tumor-resistance against cisplatinum. These tumors exhibit high levels of chromosomal instability. We have previously reported that Notch3 gene amplification occurs frequently in tumors from ovarian cancer, and overexpression of Notch3 was found in more than half of ovarian serous carcinomas. It is well-established that aberrant Notch signaling contributes to tumorigenesis. In this study, we demonstrate that Notch3 expression contributes to carboplatin resistance by providing survival signals to treated ovarian cancer cells. In addition to Notch3 receptor, we identified Jagged1 as its main ligand using both co-immunoprecipitationed and co-binding assays. Jagged1 expressed not only in ovarian cancer cells but also in peritoneal mesothelial and ovarian cancer stroma cells. Downregulation of Jagged1 in mesothelial and tumor feeder cells inhibits tumor cell adhesion and proliferation. Notch3, as well as Jagged1, in ovarian cancer cells is cleaved by a metalloprotease. In ovarian cancer cells, we identified a disintegrin and metalloproteinase 17 (ADAM17) involved in Jagged1 cleavage. Treatment of cancer cells with a metalloprotease inhibitor inhibits proliferation and induces apoptosis to a similar extent as observed with gamma-secretase inhibitors, which were used previously for treatment of other types of cancer. More importantly, treatment with these inhibitors sensitizes cells to carboplatin. Since both types of inhibitors have displayed high cytotoxicity as a side effect during the course of treatment, their use is limited. We therefore utilized a new approach by interfering with Notch signaling at the level of ligand-receptor interaction. Application of a Jagged1 fusion protein that binds to the Notch receptor and blocks access for the natural ligand leads to a decrease in proliferation and specific downregulation of Notch3 target genes. Furthermore, we developed Notch3 decoy fusion proteins that bind Jagged1, thus blocking the access for the Notch3 receptor. Taken together, our results demonstrate that interfering with the Notch3 signaling pathway at various levels provides a new therapeutic strategy in ovarian cancer and may revert the cisplatinum resistance in recurrent tumors.

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191 GSK3-Beta as a Potential Therapeutic Target in Endometrial Cancer

Premal H. Thaker, Bobbie J. Rimel, Y Yin, S. Arur, Tim Schedl, Liang Ma, Paul J. Goodfellow

Siteman Cancer Center, Washington University School of Medicine, St. Louis MO

Endometrial cancer is the most common gynecologic malignancy. Effective non-toxic therapies for advanced or recurrent endometrial cancer are lacking. Recently successful biologic therapies in other tumors have paved the way for exploring targeted therapies for endometrial cancer. Laboratory findings have proven mutational activation of the extracellular signal-regulated kinase (ERK) pathway is a frequent oncogenic event in endometrial cancers. ERK signaling is mediated by phosphorylation of substrate proteins, and to date few of the effector molecules have been identified and characterized. We have employed a 3-part functional genomics approach to identify novel evolutionary conserved ERK substrates that function in C. elegans germ cell development. One of the ERK substrates that inhibits ERK-dependent processes, GSK3b (glycogen synthase kinase 3-beta; GSK3B), was chosen for investigation as a potential therapeutic target. GSK3b's role in cancer biology has been well-established. GSK3B functions in canonical Wnt signaling, plays a critical role in NFkB signaling, and is important for cell proliferation/survival.

To assess the role of GSK3-beta in endometrial cancer cell proliferation, we inhibited GSK3b activity using either lithium chloride or AR-A014418 (a.k.a. Inhibitor VIII) in endometrial cancer cell lines AN3CA, HEC1-A, ISHIKAWA, and SPEC-2 and in EM-E6/E7 TERT CS transformed normal endometrial cell line; 50uM inhibitor was cytotoxic in all cancer cell lines, but not in the transformed cell line.

To determine the effects of inhibitor VIII on proliferation, we performed a time course experiment. Cell lines were treated with either DMSO (vehicle) or inhibitor VIII 24 hours after plating. Cell counts revealed that GSK3b inhibitor VIII inhibited cell proliferation as early as 24 hours post-treatment in all cancer cell lines, and the effect persisted for 72–96 hours. Mouse knockout studies demonstrated GSK3b is not required for cell survival/proliferation in normal cells; GSK3b null embryos can survive to midgestation, and a mutant embryonic fibroblast cell line has been established. The findings from our cancer cell line studies suggest a tumor-specific function for GSK3b in promoting cell growth and that function can be blocked by GSK3b inhibition. We have initiated experiments to assess the effects GSK3b inhibitors have in an in vivo orthotopic model of endometrial cancer. Antibodies that recognize the ERK phosphorylated form of GSK3b are being evaluated in endometrial cancer cell lines and primary tumors to determine the relationship between ERK activation, GSK3b phosphorylation status, and clinico-pathologic features.

192 Inhibition of Wnt Signaling by Pyrvinium

Curtis Thorne, Kelly Meyers, **Ethan Lee**

Vanderbilt University Medical Center

We developed a biochemical assay using Xenopus laevis egg extract that recapitulates Axin and beta-catenin turnover in response to addition of recombinant co-receptor (LRP6). In Xenopus egg extract, beta-catenin is robustly degraded and Axin is relatively stable. In the presence of recombinant LRP6, however, beta-catenin degradation is inhibited and Axin degradation is stimulated. Using this system (with beta-catenin fused to firefly luciferase and Axin fused to Renilla luciferase as reporters for protein levels), we performed a high-throughput screen to identify small molecules that reverse the effects of recombinant LRP6. From this screen, we identified an FDA-approved compound (pyrvinium) that promotes beta-catenin degradation and inhibits Axin degradation in Xenopus extract. In cultured mammalian cells, pyrvinium blocks the nuclear accumulation of beta-catenin in response to Wnt3a treatment. Using a TOPFlash reporter cell line in which luciferase is under the control of the TCF/Lef1 promoter, we found that pyrvinium inhibits Wnt signaling with an IC50 of ~10 nM. Inhibition of Wnt signaling was further confirmed by real-time RT-PCR of the Wnt target genes, Axin2 and c-MYC. Pyrvinium is also active in Xenopus laevis embryos, a well-characterized system for studying Wnt signaling in vivo. Injecting XWnt8 into early Xenopus embryos induces ectopic expression of the organizer gene, chordin, and duplication of the body axis. We found that co-injection of pyrvinium blocks the effects of XWnt8 in this assay. Surprisingly, pyrvinium inhibits Wnt signaling in the colorectal cancer lines HCT116 WTKO (non-degradable mutation in beta-catenin) and SW480 (mutation in APC). Pyrvinium is selective for mutation of the Wnt pathway as the SW480 cancer line in which wild-type APC has been introduced is much less sensitive to the effects of the drug. In preliminary studies of pyrvinium, APCmin mice injected with pyrvinium exhibit reduced cytoplasmic and nuclear beta-catenin levels in the tumors. Future goals are directed towards identifying the cellular target of pyrivnium and performing medicinal chemistry around the pyrvinium structure.

193 The Ubiquitin-Conjugating Enzyme CDC34 as an Emerging Cancer Therapeutic Target

Julia I. Toth, **Matthew D. Petroski**

Signal Transduction Program, NCI-Designated Cancer Center, Burnham Institute for Medical Research, La Jolla, CA

The Ubiquitin Proteasome System (UPS) has critical functions regulating protein homeostasis mechanisms and alterations in key regulatory enzymes (e.g., BRCA1, SKP2), and changes in the abundance of specific proteins regulated by the UPS (e.g., p53, p27, beta-catenin) are associated with cancer. The proteasome inhibitor bortezomib for multiple myeloma and mantle cell lymphoma represents the first FDA-approved drug targeting any aspect of the UPS. Whereas bortezomib in combination with other drugs appears to be very effective, its toxicity and high frequency of side effects have limited its potential. Thus, modulating pathway specific enzymes of the UPS—ubiquitin conjugating enzymes and ubiquitin ligases—may circumvent these issues and lead to improved treatment options. The complex mechanisms of action of these enzymes and their unclear physiological roles have hampered progress towards this goal.

We are currently validating the ubiquitin-conjugating enzyme CDC34 as a target for developing cancer therapeutics. Increases in the expression of CDC34 have been observed with hepatocellular carcinomas, pediatric acute lymphoblastic leukemia, and multiple myeloma. Experiments using multiple myeloma cell lines have demonstrated that over-expression of a catalytically inactive form of CDC34 enhances the effectiveness of bortezomib and other multiple myeloma drugs by inducing apoptosis. This effect is suppressed by over-expression of wild-type CDC34. We are developing biochemical and cell-based assays to screen for small molecule probes that selectively modulate CDC34. We are also characterizing the regulation of CDC34 with the goals of identifying molecular signatures of its activities in cells and alterations associated with specific cancers.

194 Dual Blockade of Lipid and Protein Kinases Potentiates Apoptosis in Malignant Glioma

William A. Weiss[1], Christine Cheng[1], Ben Houseman[1], Eli Zunder[1], C. David James[1], Scott Oakes[1], Kevan Shokat[1], Nathanael S. Gray[2], Brian Pollok[3], Qi-Wen Fan[1]

[1]University of California, San Francisco; [2]Dana-Farber Cancer Institute, Boston MA; [3]Invitrogen Corp, Carlsbad, CA

Toxicity represents a major obstacle in drug development, limiting the ability of most drugs to reach the market and impeding capital investment in cancer therapy, as failures typically occur late in development. We analyzed a toxic drug that failed preclinically, and distinguish targets critical to efficacy from those contributing non-specifically to toxicity. We use this information to develop combination therapy with existing drugs, maximizing on-target efficacy while limiting toxicity, resulting in a new combination therapy translatable to patients with glioma. In screening isoform selective inhibitors of phosphatidylinositol 3-kinase (PI3K--including chemotypes representative of most Pharma drugs), we characterized the Piramed drug PIK-75, a Piramed imidazopyridine inhibitor that induced potent cytotoxicity in glioma but that had poor drug-like properties. We present data that induction of apoptosis by this drug requires blockade of both PI3K and additional serine-threonine kinases. We therefore hypothesized that inhibitors of PI3K in conjunction with inhibitors of other critical kinases could transform a cytostatic therapy into a cytotoxic one, thereby improving outcomes in patients with glioma. PIK-75 differed from the PIK-90, a Bayer drug and specific inhibitor of PI3Kα, in inducing arrest at G2, causing apoptosis in glioma, and in driving non-specific toxicity. We demonstrate that blockade of PI3K was critical to the ability of PIK-75 to induce apoptosis and subsequently performed a kinome scan to identify all annotated kinases targeted by PIK-75 (20% of the kinome). Among these, inhibitors of cell cycle checkpoint kinases (cyclin dependent kinases 1 and 2--cdk1 and cdk2) were known to induce arrest at G2, whereas inhibition of other PIK-75 targets, including PI3Ka, should induce arrest at G1. Apoptosis induced by this combination proceeded through a Bax-dependent intrinsic pathway. We present data demonstrating that inhibition of cdk1 and cdk2 cooperated with inhibition of PI3K alpha transforming a cytostatic therapy into a cytotoxic and non-toxic approach in glioma. Using the clinical cdk1/2 inhibitor roscovotine, alone and in combination with PIK-90, we demonstrate that blockade of PI3Ka cooperated with blockade of cdk1 and cdk2 in primary human glioblastoma xenografts, inducing apoptosis in vivo. These experiments identify a novel combination therapy for glioma and provide the preclinical basis for a clinical trial to evaluate this therapy in patients with glioma.

195 Therapeutic Modulation of HSP70 as a Strategy to Enhance ER Stress-Based Therapy in Pancreatic Cancer

Matthew White[1,2], Wei Qi[1,2], Jennifer Hsing Choe[2], Gauri Varadhachary[3], James L. Abbruzzese[3], William Bornmann[4], Craig Logsdon[2], **David J. McConkey**[1,2]

Departments of [1]Urology, [2]Cancer Biology, [3]Gastrointestinal Medical Oncology, and [4]Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, Houston, TX

The endocrine and exocrine cells of the normal pancreas maintain active protein secretory programs and have highly developed protein quality control mechanisms to ensure proper ER-Golgi protein transport. We previously showed that proteasome inhibitors and certain conventional chemotherapeutic agents induce apoptosis in human pancreatic cancer cells by perturbing protein quality control, leading to ER stress, and agents that exacerbate ER stress (including inhibitors of histone deacetylases) enhance the cytotoxic effects of these agents. Because ER stress elicits a transcriptional cytoprotective response (termed the "unfolded protein response"), we compared the gene expression profiles of cells before and after exposure to the proteasome inhibitor bortezomib (Velcade). One of the top "hits" was the protein chaperone HSP70, a gene that was also identified as one of the most strongly upregulated in multiple myeloma (MM) cells exposed to the drug. Upregulation of HSP70 was confirmed by real-time PCR and immunoblotting. Chemical inhibitors of HSF-1 (quercetin, KNK-437) or shRNA-mediated knockdown of HSP70 enhanced bortezomib-induced cell death. Results of experiments designed to identify the transcription factor(s) involved in bortezomib-induced HSP70 upregulation and the effects of HSP70 inhibition on the growth of orthotopic human pancreatic tumors will be presented at the meeting. Overall, our data demonstrate that HSP70 functions to inhibit ER stress-induced death in human pancreatic cancer cells. Chemical inhibitors of HSP70 may be uniquely capable of enhancing the cytotoxic effects of bortezomib and other agents (cisplatin, HSP90 inhibitors) that upregulate HSP70 as a cytoprotective response.

196 Development of Novel Molecular Agents That Inhibit Pathways Associated With Nitric Oxide Synthase and Cycloxygenase-2 High Expression and Poor Prognosis in Breast Cancer

David A. Wink[1], Sharon Glynn[2], Christopher Switzer[1], Lisa Ridnour[1], Stefan Ambs[2]

[1]Radiation Biology Branch, [2]Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, MD

Translational research has showed that the inflammatory proteins iNOS and COX-2 are indicators of poor prognosis in many cancers. We have been studying the role of nitric oxide (NO) and other reactive species in cancer biology. These studies have shown that specific concentrations of NO determine its pro- or anti-tumorigenic effects of this molecule. With prolonged exposure of breast cancer cells to μM amounts of NO, there is an increase in the phosphorylation of p53 and cancer cell cytostasis. However, when cells are exposed to 100 nM of NO, there is predominant activation of in pro-tumorigenic molecular pathway activation such as MAPK, Akt-P, and HIF1α. Several of these pathways are also activated by PGE2. Studying estrogen receptor negative [ER(-)], we observed that both iNOS and COX-2 were frequently upregulated, and their increased expression was associated with decreased breast cancer survival. When expressed simultaneously, there is a dramatic increase in patient mortality. From these data, we have been able to develop cellular models to determine if novel anticancer compounds can reverse the poor outcome phenotypes associated with iNOS and COX-2. This research discovered a class of thiol-based compounds that activates a tumor suppressor protein and reverses the molecular phenotype associated with iNOS and COX-2 in ER negative breast cancer. Exposure of cells to these compounds results in increased expression of the tumor suppressor gene, PP2A, coupled with reduced levels of Akt-P and c-Myc, resulting in reduced proliferation. In addition, these compounds result in increased expression of E-cadherin and decreased expression of vimentin, with corresponding morphological changes indicative of mesenchymal to epithelial transition. These observations suggest that these compounds may reverse some of the processes associated with metastasis. Additionally, the regulation of E-cadherin is associated with Akt/MAPK and HIF pathways, which, as previously described, are inhibited by these thiol complexes. This presentation will focus on understanding the chemical biology of nitric oxide and how it increases cancer risk. Furthermore, we will describe potential new molecular targets for the treatment of cancer. In our presentation, we will focus on understanding the chemical biology of nitric oxide and how it influences breast cancer survival and describe potential new molecular targets for the treatment of ER-negative breast cancer.

197 The Notch Ligand Jagged2 Promotes Metastasis Through a GATA3/miR-200 Regulatory Loop

Yanan Yang[1]*, Young-ho Ahn[1]*, Don L. Gibbons[1]*, Yi Zang[1], Wei Lin[1], Anindya Samantha[1], Nishan Thilaganthan[1], Daniel C. Moreira[1], Chad J. Creighton[2], Gregory J. Goodall[3], **Jonathan M. Kurie**[1] (*equal contributors)

[1]Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX; [2]Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX; [3]Centre for Cancer Biology, Hanson Institute, Adelaide, Australia and Discipline of Medicine, The University of Adelaide, Adelaide, Australia

Strategies to detect metastasis-prone tumor cells and to inhibit their spread are of critical importance to the improvement of clinical outcome for cancer patients. Treatment approaches for metastatic epithelial cancers have focused on disease eradication, which have failed owing to the outgrowth of drug resistant tumor cells. An alternative approach is metastasis prevention to allow the patient to co-exist with the primary tumor. Metastatic disease originates from populations of tumor cells that have the propensity to undergo epithelial-to-mesenchymal transition (EMT), but the signals that initiate EMT in these cells have not been fully defined. Here we addressed this question in mice that develop metastatic lung adenocarcinoma owing to expression of mutant K-ras and p53. We found that metastases arose from a population of tumor cells that had high expression of Notch ligands. Jagged2 promoted EMT by a Notch-dependent suppression of microRNA-200 (miR-200), a family of miRs that negatively regulates ZEB transcriptional repressors. We identified an upstream miR-200 promoter element that is a direct target for GATA3 and, reciprocally, GATA3 mRNA sequences that are direct targets for miR-200, indicating that a double-negative feedback loop tightly controls the levels of GATA3 and miR-200. Abrogating miR-200 suppression through knockdown of JAG2 or GATA3 blocked metastasis. We conclude that Notch promotes metastasis by activating a GATA3/miR-200 regulatory loop and speculate that Notch may be an efficacious target for metastasis prevention.

198 Correlation Between Polymorphisms in the Reduced Folate Carrier Gene (SLC19A1) and Survival After Pemetrexed-Based Therapy in Non-Small Cell Lung Cancer (NSCLC): An NCCTG N0026 Based Study

Alex A. Adjei[1], Araba A. Adjei[1], Oreste E. Salavaggione[1], Sumithra J. Mandrekar[2], Grace K. Dy[1], Katie L. Allen Ziegler[2], Chiaki Endo[3], Julian R. Molin[2], Steven E. Schild[4]

[1]Roswell Park Cancer Institute, Buffalo, NY; [2]Mayo Clinic, Rochester, MN; [3]Tohoku University, Aoba-ku, Sendai, Japan; [4]Mayo Clinic, Phoenix, AZ

Background: Pemetrexed is a multi-targeted antifolate whose intracellular accumulation depends primarily on activation/inactivation processes by folylpolyglutamate synthetase (FPGS) and gamma glutamyl hydrolase (GGH). The predominant mechanism of pemetrexed resistance results from alterations in FPGS activity. Additionally, increased expression of GGH is associated with resistance to antifolates. Other mechanisms of resistance include changes in transport by the reduced folate carrier (SLC19A1). We hypothesized that polymorphisms in these genes may correlate with clinical response and/or toxicity of pemetrexed. We used our resequencing data from 60 Caucasian DNA samples from the Coriell Cell Repository to derive haplotype (ht) and LD-tag SNPs for FPGS. We utilized the NCBI variation database and the HapMap database to obtain additional polymorphism information on GGH and SLC19A1. Data from a phase II trial of gemcitabine and pemetrexed were utilized for genotype to phenotype studies. All patients with available DNA were genotyped for the selected polymorphisms in FPGS, GGH and SLC19A1. Efficacy and adverse event outcomes were compared between the genotype variant subgroups. **Results:** Fifty-four patients had genotype results for all polymorphisms studied. Patients with the homozygous variant genotypes for SLC19A1 IVS4(2117) C>T and IVS5(9148) C>A and wild-type genotype for Exon6(2522) C>T had a significantly better overall survival (OS) compared to their counterparts (median OS in months: 8.9 (CC) vs. 14.0 (TC) vs. 16.7 (TT); 9.4 (CC) vs. 10.3 (AC) vs. 22.7 (AA) and 22.7 (CC) vs. 10.3 (CT) vs. 9.4 (TT); all log rank p=0.03). Patients with the heterozygous TC genotype for GGH IVS5(1042) T>C polymorphism had better clinical benefit (confirmed response + stable disease) rate compared to the TT genotype (85% vs. 60%; Odds ratio (OR) = 4.0; p=0.06). Greater risk for grade 3/4 SGPT (ALT) elevation was observed in patients who were heterozygous (GA) for the FPGSIVS1(28) G>A polymorphism (43% vs. 13%; OR=5.0, p=0.07). **Conclusion:** Our findings indicate that polymorphisms in SLC1A91 could possibly predict for overall survival differences in pemetrexed-treated NSCLC. Additionally, polymorphisms in GGH and FPGS show marginal associations with response and AE outcomes. Further work to validate these results in larger prospective studies of pemetrexed is warranted.

199 Discovery of Novel Molecular Profile of Response to Cetuximab Using a Phosphoproteomic Approach

Aarif Ahsan[1], Xiaoping Ao[1], David M. Lubman[2], Theodore S. Lawrence[1], **Mukesh K. Nyati**[1]

Departments of [1]Radiation Oncology and [2]Surgery, University of Michigan, Ann Arbor, MI

With recent discoveries into the genetics of cancer, many new targets for cancer treatment have been identified. One of these targets is the EGFR, which is amplified or overexpressed in various types of cancers, including those of head and neck origin. Along with genetic discoveries, several new agents have been developed in an effort to target these molecules with the hope of achieving cure without the toxicity typically associated with traditional chemotherapeutic agents. One such agent, cetuximab, has been proven effective in local and metastatic head and neck cancers in combination with cisplatin or radiation. Although these results are encouraging, still only one out of four patients treated with cetuximab in combination with radiation or cisplatin benefits from this novel and very expensive treatment. We feel it is of great importance to differentiate between patients who are likely to respond to cetuximab and those who will not. EGFR immunostaining in pre-treatment patient specimens, as well as by analyzing EGFR gene copy number, have failed to predict response to EGFR inhibition therapy. We hypothesize that through phosphoproteome analysis, we can identify alterations in novel signal transduction molecule(s) induced by cetuximab treatment that will predict subsequent response to cetuximab. To this end, we assessed phosphoproteomic changes upon cetuximab treatment in UMSCC-1 (cetuximab-responsive) and UMSCC-74 (cetuximab-non-responsive) cell lines. We found pharmacodynamic changes in over 12 novel proteins upon cetuximab treatment in UMSCC-1 cell line. We have initially investigated three novel proteins (NCoR1, MeCP2 and MBD2), as they are part of the large family of methyl-CpG binding proteins and cause transcriptional repression (NCoR1 and MeCP2) or activation (MBD2) by immunoprecipitating total proteins followed by immunoblotting with phospho-specific antibodies. Using this strategy, we have confirmed (a) these three proteins are indeed phospho proteins, and (b) in UMSCC-1 cells phosphorylation of both NCoR1 and MeCP2 is induced, whereas phosphorylation of MBD2 is decreased, confirming phosphoproteomic data. Importantly, in UMSCC-74B we found that cetuximab caused either no effect or an opposite effect to that seen in the sensitive cells. These data suggest that we have a powerful strategy for finding novel biomarkers of response to cetuximab. These biomarkers and additional biomarkers that we hope to discover will undergo rigorous in vitro and in vivo testing before we employ them on TMA that we are currently generating from a xenograft study.

200 UPR Activation Decreases Apoptotic Response of Cells to Etoposide-Induced DNA Damage

Walid Awad, Melissa Mann, **Linda M. Hendershot**

St. Jude Children's Research Hospital, Memphis, TN

Poorly vascularized solid tumor cells encounter a restricted supply of nutrients and oxygen, which can activate cellular stress response pathways including the unfolded protein response (UPR). The UPR is largely a cytoprotective response that allows cells to survive conditions that alter normal protein folding in the endoplasmic reticulum. This response is proximally regulated by three ER localized, transmembrane proteins: Ire1, PERK, and ATF6. Together these three proteins regulate specific components of the response. Recent data have demonstrated that UPR activation occurs in a number of different types of human tumors and has been shown to be critical to tumor growth in mouse xengraft models. In addition, activation of the UPR in cultured cells can dramatically affect their sensitivity to chemotherapeutic agents. We have shown previously that UPR activation is both necessary and sufficient to decrease topoisomerase IIα protein levels and to render cells resistant to etoposide, a topoisomerase II-targeting drug. Using mouse embryonic fibroblasts that are deficient in the various UPR signal transducers, we found that loss of topoisomerase IIα is downstream of Ire1 activation but is not dependent on XBP-1, making this a unique Ire1 function. Surprisingly, Ire1 did not contribute significantly to etoposide resistance. Although loss of PERK did not affect changes in topoisomerase II α protein levels in response to UPR activation, it did significantly decrease the resistance to etoposide that is observed in response to ER stress. These data argue that the increased resistance to etoposide is not primarily due to decreased topoisomerase IIα levels and that a component of the PERK signaling branch provides this protection. PERK is an eIF-2α kinase that globally regulates protein translation and specifically induces the ATF4 transcription factor. To further explore the pathway downstream of PERK, we examined ATF4 control and knockout cells, which revealed that the altered chemosensitivity, although dependent on the PERK branch, is upstream of ATF4, suggesting that the effect is due to translation inhibition. UPR activation does not alter drug levels in the cells or prevent DNA damage but does appear to affect the DNA damage signaling pathway. Studies are underway to determine where the breakdown in this pathway occurs.

201 Nucleotide Excision Repair Pathway Gene Haplotypes and Response to Platinum Based Therapies in Squamous Cell Carcinoma of the Head and Neck

Shama Buch, Shilong Zhong, **Marjorie Romkes**

University of Pittsburgh, University of Pittsburgh Cancer Institute, Pittsburgh, PA

Squamous cell carcinoma of the head and neck (SCCHN) represents 4.5% of the incident cancers in the Unites States each year and is the sixth most common cancer worldwide. Despite being a potentially curable malignancy in its early stages, the majority of patients present with locally advanced disease (stages III–IV) and will die within 2 years of diagnosis after treatment with standard approaches. Platinum-based therapy has historically been considered an important chemotherapeutic regimen for SCCHN, particularly in combination with radiotherapy. The nucleotide excision repair (NER) pathway has been implicated as an important factor in modulating overall cancer survival and responsiveness to platinum based chemotherapy agents in a variety of tumor types. We have developed a SNP selection strategy for screening DNA repair pathway haplotypes based on genotyping 384 SNPs using a custom designed Illumina GoldenGate assay. Our SNP selection strategy incorporates haplotype tagSNPs, functional SNPs characterized by amino acid substitutions, evolutionary conservation, thermodynamic tolerance, published epidemiological data, and a minimum minor allele frequency (MAF) of 10%. The association with progression free survival (PFS) was evaluated for each SNP in the 384 SNP panel in cases treated with adjuvant platinum therapy (n=124). One or more polymorphisms in ARF, CDK7, ERCC3, P53, RAD23B, and RPA3 were significantly associated with PFS with adjustment for age, gender, smoking, and stage and correction for multiple testing based on false discovery rate. The analysis was extended to haplotypes for each gene in the 384 SNP panel. Haplotypes were reconstructed for each gene and association with PFS, and time to second event was evaluated using the Cox proportional hazards model. The most frequent haplotype was used as the referent group for the estimation of hazard ratios. Hazard ratios were calculated for common haplotypes (i.e., those that were present at a frequency greater than 2%) and adjusted for age, gender, smoking, and stage. Among these platinum-treated SCCHN cases, two gene haplotypes (E2F1 and P53) were associated with enhanced PFS, while six other gene haplotypes were associated with reduced PFS. These results support the hypothesis that DNA repair pathways represent important cellular processes relevant to variation in disease survival and treatment response, and our results are currently being validated in a larger study population.

202 Germline Variation in Complement Genes and Event-Free Survival in Follicular Lymphoma

James R. Cerhan[1], Matthew J. Maurer[1], Anne J. Novak[1], Stephen M. Ansell[1], William R. Macon[1], Susan L. Slager[1], Brian K. Link[2], George J. Weiner[2], Thomas E. Witzig[1], Thomas M. Habermann[1]

[1]College of Medicine, Mayo Clinic, Rochester, MN; [2]College of Medicine, University of Iowa, Iowa City, IA

Background: Complement plays a central role in innate immunity and also functions as a regulator of the overall immune response, including T-cell responses. We recently reported that germline genetic variation in C2, C5, C7, and C9 was associated with risk of developing non-Hodgkin lymphoma (Br J Haematol 2009;145:614). Here we evaluate whether complement genes are associated with event-free survival (EFS) in follicular lymphoma. **Methods:** We genotyped 166 single nucleotide polymorphisms (SNPs) from 32 complement pathway genes in a prospective cohort of 115 newly diagnosed follicular lymphoma patients enrolled at the Mayo Clinic from 2002–2005. All patients were systematically followed through 2008 for EFS (defined as disease progression, retreatment, or death due to any cause). Tagging and nsSNPs were selected from HapMap. Cox regression was used to estimate Hazard Ratios (HRs) for individual SNPs with EFS. The most prevalent homozygous genotype was used as the reference group, and each polymorphism was modeled individually as having a log-additive effect in the regression model. For gene-level analyses, we used a principal components (PC) based gene-level test. All models were adjusted for sex, FLIPI, follicular grade III, and initial treatment. **Results:** The median age at diagnosis was 61 years (range, 25–85). Some 57 (50%) of the patients had an event, at a median followup of 59 months (range, 33–73) for living patients. In the PC gene analyses, CFH ($p=0.004$), CD55 ($p=0.02$), CFHR5 ($p=0.02$), and CFHR1 ($p=0.02$) were significant at $p<0.05$, and these genes had noteworthy q-values after consideration of multiple testing ($q=0.04$ for CFH and $q=0.08$ for CD55, CFHR5, and CFHR1). For CFH, 2 of the 11 tagSNPs (rs1329423, rs3766404) were significant at $p<0.05$ as was the nsSNP rs1065489 (E936D), HR=0.39 ($p=0.004$). SNPs that were statistically significant ($p<0.05$) in the remaining genes included both tagSNPs (rs4844591, rs2564978) for CD55; three of five tagSNPs (rs3748557, rs12092294, rs6694672) for CFHR5; and the only tagSNP (rs436719) for CFHR1. **Conclusions:** Genetic variation in CFH, CD55, CFHR5, and CFHR1 was associated with EFS in follicular lymphoma after adjustment for clinical variables. These genes are part of a gene cluster at 1q32-q32.1 involved in the regulation of C3. CD55 encodes a membrane complement regulatory protein that can allow tumor cells to evade complement attack. These results are being validated in a second set of SPORE patients.

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203 Understanding and Targeting Cancer Stem Cells

Curt Civin[1,2], Saul Sharkis[2], Robert Brodsky[2], Richard Jones[2], Donald Small[2], Alan Friedman[2], Steven Goodman[2], Mark Levis[2], William Matsui[2], Sarah Wheelan[2]

[1]Greenebaum Cancer Center, University of Maryland School of Medicine; [2]Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine

As we identify and isolate normal tissue-specific stem cells and Leukemia Stem Cells (LSCs), we are assessing the activity and role of critical molecular signaling pathways in normal stem-progenitor cells and LSCs. For example, our Program Project Grant studies include the following directions:

- Separate LSCs using properties that distinguish normal tissue-specific stem cells from their differentiated progeny. In addition, we will investigate whether the divergent outcomes between “pediatric-type” and “adult-type” acute lymphoblastic leukemia (ALL) are the result of different stem cell populations. In clinical trials, we will test whether targeting ALL stem cells via inhibition of the hedgehog pathway (e.g., Smothened) or telomerase will improve the outcome in adult ALL.
- Determine if the FLT3 mutation is expressed in LSCs from patients with FLT3 mutant acute myeloid leukemias (AMLs). In addition, we will evaluate whether LSCs from patients with FLT3 mutant AML are heterogeneous with respect to response to in vivo treatment with FLT3 inhibitors. We will also study how other oncogenes (known to occur in association with mutated FLT3 in human leukemia cases) cooperate with mutant FLT3 to transform normal hematopoietic stem-progenitor cells into LSCs. The understanding gained may influence design of future clinical trials employing FLT3 inhibitors.
- Quantify microRNA expression at defined steps of normal human and mouse hematopoietic development, and in LSCs versus the bulk populations of leukemia cells in a given patient sample. In addition, we will determine the cell and molecular mechanisms by which selected microRNAs affect the differentiation and biology of primary human hematopoietic stem-progenitor cells and LSCs. This understanding may inspire clinical trials of microRNA mimics or microRNA antagonists or of drugs directed at pathways regulated by microRNAs.

204 Genetic Variations in MicroRNA Genes and Risk of Oral Premalignant Lesions

Jessica Clague[1], Scott M. Lippman[2], Hushan Yang[1], Michele A. Hildebrandt[1], Jack Lee[3], **Xifeng Wu**[1]

Departments of [1]Epidemiology, [2]Thoracic/Head and Neck Oncology, and [3]Biostatistics, The University of Texas M. D. Anderson Cancer Center, Houston, TX

miRNAs have been reported to play a key role in oncogenesis, and recently studies have looked at the role miRNAs might play in the risk of premalignant lesions. To our knowledge, no study has investigated the association between miRNA pathway genetic variations and risk of oral premalignant lesions (OPL). We genotyped 31 single nucleotide polymorphisms (SNPs) among 21 miRNA-related genes, including miRNA biogenesis gene and miRNA gene, in a case-control study including 136 OPL patients and 136 matched controls. For pri-mRNA SNP rs7372209 in mir26a-1, patients with at least one variant allele had a significantly increased risk of OPL (OR, 2.09; 95% CI, 1.23–3.56). Likewise, patients with at least one variant allele of miRNA biogenesis gene DICER:rs3742330 had a significantly increased risk of OPL (OR, 2.09, 95% CI, 1.03–4.24). To assess the cumulative effects, we performed a combined unfavorable genotype analysis that included all SNPs showing at least a borderline statistical significance in individual analysis. Compared to the low-risk group with ≤1 unfavorable genotypes, the OR for the medium risk group with two or three unfavorable genotypes was 3.52 (95% CI, 1.81–6.84), and the OR for the high risk group with ≥4 unfavorable genotypes increased to 21.35 (95% CI, 5.08–89.79; P for trend < 0.0001). This study presents the first epidemiologic evidence supporting that individual as well as combined genotypes of miRNA-related variants may be used to predict the risk of OPL. Future studies of large sample sizes are warranted to validate these findings and also to assess the role of these SNPs in the malignant progression of OPL. The validated SNPs may be useful for identifying patients with OPL at high risk for progression to oral cancer.

205 KLF4 and Notch1 as Breast Cancer Biomarkers

Andra Frost[1], Zhaoli Liu[2], Sarah Bailey[3], Kirby I. Bland[4], Albert F. LoBuglio[3], J. Michael Ruppert[5], **Susan M. Lobo-Ruppert**[3]

Departments of [1]Anatomic Pathology and [2]Cell Biology, [3]Division of Hematology Oncology, [4]Department of Surgery, University of Alabama at Birmingham; [5]Department of Biochemistry, West Virginia University

KLF4 is a zinc finger transcription factor that can function as a context-dependent tumor suppressor or oncogene. It is also one of four genes that can together reprogram differentiated adult cells into pluripotent stem cells. KLF4 functions as an oncogene by binding to the Notch1 promoter and upregulating Notch1 transcription. Active Notch1 is required as gamma secretase inhibitors and siRNA-mediated suppression of Notch1 abrogate transformation by KLF4. However, dominant negative inhibitors of the canonical Notch1 signaling pathway do not block transformation by KLF4, indicating that Notch1 may signal via an alternative pathway downstream of KLF4.

KLF4 and Notch1 were each previously reported to be predictors of outcome in breast cancer. We now show that Notch1 and KLF4 expression may together be better predictors of outcome than either marker alone. Because Notch1 transcription is modulated by KLF4, tumors co-expressing these proteins may be clinically distinct in their response to Notch inhibitors or to other therapies.

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206 A Mitochondrial Target Sequence Polymorphism in the Manganese Superoxide Dismutase Gene Predicts Inferior Survival in Breast Cancer Patients Treated With Cyclophosphamide

Sharon A. Glynn[1], Brenda J. Boersma[1], Tiffany M. Howe[1], Hege Edvardsen[2], Stephanie B. Geisler[3], Julie E. Goodman[4], Lisa A. Ridnour[1], Per E. Lenning[3], Anne-Lise Børresen-Dale[2], Bjorn Naume[2], Vessela N Kristensen[2], Stephen J. Chanock[1], David A. Wink[1], **Stefan Ambs**[1]

[1]National Cancer Institute, Bethesda, MD; [2]Institute for Cancer Research, Norwegian Radium Hospital, Oslo, Norway; [3]Institute of Medicine, University of Bergen, Norway; [4]Gradient Corporation, Cambridge, MA

Background and Hypothesis: Manganese superoxide dismutase (MnSOD) protects against oxidative damage and modulates the efficacy of chemotherapeutic drugs. A functional single nucleotide polymorphism (SNP) in codon 16 of SOD2 (rs4880), which encodes MnSOD, results in a substitution of valine by alanine (Val16Ala). We hypothesized that this SNP affects breast cancer survival of patients receiving chemotherapy and would be a useful biomarker for prediction of therapy response. **Experimental Design:** Two patient populations from the United States (n=248) and Norway (n=340) were genotyped for Val16Ala. Kaplan-Meier survival and Cox Proportional-Hazards regression analyses were used to examine the relationship between Val16Ala and disease-specific survival and response to therapy. **Results:** Val16Ala was significantly associated with breast cancer outcome in both patient populations. Carriers of the Ala allele had inferior survival rates in the multivariate analysis [Hazard ratio (HR) = 2.44; 95% confidence interval (CI), 1.11–5.37 in United States cohort and 1.91; 95% CI, 1.06–3.45 in Norway cohort for Ala/Ala versus Val/Val]. In an analysis of the combined cohorts, this association was significant for patients receiving adjuvant therapy (HR = 2.47; 95% CI, 1.46–4.19) but not for patients without it (HR = 1.47; 95% CI, 0.57–3.74). After further stratification by type of chemotherapy, the effect of the Ala allele was mostly restricted to cyclophosphamide-containing chemotherapy regimens. Patients with the Ala/Ala genotype who received this therapy had a significantly worse survival than those with the Val/Val genotype (HR = 22.0; 95% CI, 5.22–92.9; Ala/Ala versus Val/Val). **Conclusion:** The Val16Ala polymorphism affects survival of patients receiving cyclophosphamide-containing chemotherapy. The finding provides the first evidence pointing toward a mechanism for cyclophosphamide-resistance in breast cancer patients and may have important clinical implications because 20% to 25% of the general population in the United States and Europe is carrying this genotype. Although preliminary, these data suggest that patients with the Ala/Ala genotype should be considered for alternative treatment.

207 Efficacy of Combined Vandetanib, Docetaxel and Radiation Therapy in Human HNSCC Xenografts: Effect of EGFR Status and Utilization of Pharmacokinetic-Directed Dosing

Daniel L. Gustafson[1,2], Erica L. Bradshaw-Pierce[2]

[1]Animal Cancer Center, Colorado State University, Fort Collins, CO; [2]University of Colorado Comprehensive Cancer Center, University of Colorado, Denver, Aurora, CO

Vandetanib (ZD6474; Zactima®) is a multi-target tyrosine kinase inhibitor (TKI) with activity against VEGFR2 and EGFR, thus exhibiting both antiangiogenic as well as direct antitumor effects. The pre-clinical development of TKIs often involves screening for activity in combination with cytotoxic therapies as well as the analysis of sensitive versus resistant cell lines to determine underlying molecular pathways indicative of response. The anti-EGFR activity of vandetanib dominates when human head and neck squamous cell carcinoma (HNSCC) cell lines are screened for anti-proliferative effects resulting in response similar to gefitinib. However, studies in xenograft models have shown that vandetanib can enhance anti-tumor response of radiation therapy (RT) even in HNSCC xenografts completely refractory to vandetanib therapy alone (UMSCC10) as well as in responsive HNSCC xenografts (UMSCC2). Since docetaxel (DTX) combined with RT is a common treatment for HNSCC and the addition of EGFR inhibitors to HNSCC therapy has shown promise, we determined the effects of pharmacokinetic-directed, clinically-equivalent dosing of vandetanib and DTX combinations with RT utilizing EGFR-positive (UMSCC2) and EGFR-null (UMSCC10) HNSCC xenografts. The purpose of these studies was to determine: (1) whether only EGFR-positive tumors benefit from vandetanib addition to treatment combinations; (2) the combined effects of therapies at putative clinical doses for vandetanib and DTX; and (3) whether vandetanib/RT combinations were equivalent to DTX/RT combinations in these xenograft models to support testing of this potentially less toxic therapeutic combination. The results from these studies show that vandetanib can enhance the effects of RT and RT/DTX in both UMSCC2 and UMSCC10 tumors and that robust anti-tumor response can be observed utilizing drug doses that mimic human exposures at weekly 30 mg/m2 for DTX and 100–300 mg daily doses of vandetanib. Vandetanib/RT versus DTX/RT treatment combinations showed equivalent effects in the UMSCC2 and UMSCC10 tumors. The results from these studies show that both EGFR-positive and EGFR-null tumors show enhanced response when vandetanib is combined with DTX and RT at clinically-relevant drug doses. Further, the results from these studies suggest that vandetanib/RT is equally efficacious as DTX/RT and vandetanib/DTX/RT in EGFR-positive and EGFR-null tumors and that potential decreased toxicity of RT combinations lacking a taxane component may warrant further study.

208 Molecular Response and Imaging-Based Combination Strategies for Optimal PDT

Tayyaba Hasan[1], Stephen P. Pereira[2], Brian W. Pogue[3], Edward V. Maytin[4]

[1]Massachusetts General Hospital and Harvard Medical School, Boston MA; [2]University College London Medical School, London, UK; [3]Dartmouth College, Hanover, NH; [4]Cleveland Clinic Foundation, Cleveland, OH

The underlying hypothesis of this program is that the complexity of cancer dictates that, compared to single modalities, combination therapies that are mechanistically independent and that are directed at non-overlapping molecular targets will additively or synergistically enhance treatment outcome. Our program proposes new photodynamic therapy (PDT)-based combination treatments for pancreato-biliary tract cancer (PCBC) in an approach we term Combination Photodynamic Biologic Therapy (CPBT). PDT is a photochemistry-based modality approved for a number of cancer and noncancer pathologies and has shown promise for the treatment of PCBC, where other approaches have failed. This work builds on recent advances in the understanding of cancer biology, mechanisms of current and emerging therapies, as well as the enormous progress made in imaging technologies. Strategically, we are exploring these treatment response-enhancing CPBTs by administering a second treatment specifically tailored to a particular molecular response elicited by PDT. This work is being conducted simultaneously in clinical and pre-clinical settings. In clinical studies, we are evaluating the ability of PDT to improve the survival and quality of life for patients with PCBC. In parallel, we are conducting preclinical studies to target molecular responses that are elicited by PDT to design and optimize new clinically relevant CPBTs, such as combining PDT with Erbitux-based inhibition of the epidermal growth factor receptor. Leveraging the multidisciplinary nature of this program, we are utilizing advanced optical imaging platforms to quantitatively monitor molecular responses to treatment, such as vascular endothelial growth factor (VEGF). Additionally, we will use optical imaging as a tool for light and photosensitizer dosimetry, which will improve treatment planning and therapeutic outcome. We envision that these longitudinal imaging studies will be integrated into the development of molecular-based combination therapies for standard clinical procedures. The program is supported by one administrative and two scientific cores, which provide a central platform access for meetings, imaging and other technological advances, and for the transfer of developed technology to industry. It is anticipated that such rational, mechanism-based treatments will significantly benefit patient survival and, combined with real-time imaging to monitor tumor progression and treatment response, provide patient-specific treatments. This research will have significant impact on the clinical outcomes for patients with PCBCs, two deadly diseases that currently have few treatment options.

209 The Microenvironment of Prostate Cancer Exhibits Numerous Differential Expression Changes That Are Useful for Diagnosis Without Tumor Cells

Zhenyu Jia[1], Yipeng Wang[1], Anne Sawyers[1], Huazhen Yao[1], Anne Simoneau[1], Frank Meyskens[1], Steve Goodison[2], Michael McClelland[3], **Dan Mercola**[1]

[1]University of California at Irvine; [2]Anderson Cancer Center, Orlando, FL; [3]Vaccine Research Institute of San Diego

We have developed a linear combination model of prostate tissue that describes gene expression changes as a sum of contributions of the four major cell types that occur in tumor-enriched samples including tumor cells, stroma cells, epithelial cells of BPH, and dilated cystic glands. When combined with knowledge of the percent cell type distribution as estimated by pathologists, the model provides estimates of gene expression for each cell type. By comparing the expression of stroma cells in 18 normal volunteer biopsy samples with an equal number of tumor-adjacent stroma samples of PCa cases, we derived over 900 (out of ~ 22,000 possible) probe sets with significant gene expression differences. These were filtered by taking advantage of our knowledge of cell-specific gene expression to remove all genes that were expressed in epithelial cells at greater that 10% of stroma cells, leading to 28 stroma-specific significant differences. Further training using the 10-fold cross-validation procedure of PAM (Prediction Analysis of Microarray) confirmed the 28 gene set as a classifier with 91% accuracy. The classifier was then tested on multiple independent prostate samples, including 65 tumor cases measured on U133A Affymetrix publicly available arrays, 79 published tumor cases also measured on U133A, and 55 independent cases measured on U133plus2 arrays, which yielded diagnosis accuracies of 96–100% for the three sets. To exclude performance that may be based on recognition of tumor cells, we tested the classifier on 9 additional independent normal volunteer biopsy cases and 12 rapid autopsy cases, which yielded a nominal diagnostic accuracy of 90%. Two positive rapid autopsy cases were subsequently found to have one or more foci of occult PCa. Finally, 77 cases of manually microdissected tumor-adjacent stroma samples from cases not used in training were examined, leading to an accuracy of 94%. Twenty-eight available stroma samples from the contralateral lobe of the same tumor-bearing cases yielded a diagnosis of tumor stroma in 52%, a significant decrease, indicating that distance is a factor for the expression of the stroma-specific diagnostic genes. The results indicate that the microenvironment of PCa exhibits numerous differential gene expression changes, a subset of which may be used to identify tumor-adjacent stroma with high accuracy. The results may be applicable to the many clinical cases with an equivocal biopsy reading, as these cases commonly contain ample stroma at the sites suspicious for tumor, or may have application to biopsies read as negative but regarded with high clinical suspicion for prostate adenocarcinoma.

210 Identification of KCCN4 Upregulation in Ovarian Cancer and Its Pharmacologic Activation to Increase Sensitivity to Cisplatinum

Divya Khaitain[1], Deepak Nagendra[2], Yutaka Shoji[2], Nagendra Ningaraj[1], **John I. Risinger**[2]

[1]Curtis and Elizabeth Anderson Cancer Institute, Memorial Health Medical Center, Savannah, GA; [2]Department of Obstetrics, Gynecology and Reproductive Biology, College of Human Medicine, Michigan State University, Grand Rapids, MI

Advanced stage ovarian cancers have an extremely poor prognosis. While many respond initially to conventional chemotherapies including cis-platinum, most of these cancers recur. We identified that the KCNN4 gene was upregulated in about 75% of advanced serous carcinomas, the most common histologic type of epithelial ovarian cancer. KCNN4 forms the intermediate conductance Ca2+-activated K+ channel (IKCa channel), which serves as a highly sensitive environmental sensor of intercellular calcium. Studies in other tumor types suggest that potassium channels are integral components of cellular proliferation. RNAi knockdown of KCNN4 inhibited cell proliferation in vitro cell culture models of ovarian cancer, which suggests an essential role for KCNN4 in ovarian cancer cell growth and viability. We hypothesized that by pharmacologically activating these channels we could further increase the toxicity of ovarian cancer cells to cis-platinum. Furthermore, activators of IKca channels sensitized ovarian cancer cells to cisplatinum-induced cell death. Specifically, we found that 9 of 11 ovarian cancer cell lines demonstrated functionally active IKCa channels, and we were able to increase the sensitivity of these cells to cisplatinum following IKCa channel activation with 1-EBIO. The chemo-sensitizing effect of treating these cells with the activator was reversed when we added clotrizamole, a specific inhibitor of the IKCa channels, suggesting this effect was mediated through KCNN4. Furthermore, co-treatment of OVCAR5 cells with 1-EBIO and a non-lethal dose of cisplatinum resulted in apoptotic cell death, suggesting a chemo-sensitizing role for IKCa channels. Preliminary data in vivo mimic the in vitro results. Our data show that KCNN4 is integral to ovarian cancer cell growth and that sensitization with pharmacologic activators such as 1-EBIO may have future therapeutic benefit by increasing cancer cell sensitivity to chemotherapeutics.

211 Differential Expression of MicroRNAs in Tamoxifen-Sensitive Versus Tamoxifen-Resistant Human Breast Cancer Cells

Carolyn M. Klinge[1], Nalinie S. Wickramasinghe[1], Tissa T. Manavalan[1], Susmita Datta[2], and Theodore S. Kalbfleisch[1]

[1]Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine; [2]Department of Bioinformatics and Biostatistics, School of Public Health and Information Sciences,University of Louisville

Select changes in microRNA (miRNA) expression correlate with diagnostic markers used in early stage breast cancer therapies (e.g., estrogen receptor alpha [ERalpha]). Comparatively little is known about miRNA regulation or how antiestrogens (e.g., tamoxifen [TAM]), regulate the expression of miRNAs in breast cancer cells. The goal of our research is to determine the identity and function of miRNAs whose expression is differentially regulated by estradiol (E2) and TAM in antiestrogen/tamoxifen-sensitive versus -resistant breast cancer cell lines and to correlate these miRs and their gene targets with those dysregulated in human breast tumors, thus offering new biomarkers to be tested in patient prognosis and treatment planning. We used miRNA microarray analysis to identify miRNAs differentially expressed and regulated by E2 and 4-hydroxytamoxifen (4-OHT) in MCF-7 tamoxifen-sensitive, estrogen-dependent versus LY2 tamoxifen/endocrine-resistant human breast cancer cells. Four separate experiments were performed for each treatment group. Bioinformatic analyses to impute the biological significance of the identified miRNAs by identifying their computationally predicted target genes in the human genome using TargetScan, PicTar, and the Sanger miRBase Targets databases were performed. Additionally, we compared global miRNA and mRNA expression patterns in 4-OHT-treated MCF-7 cells to identify key targets. We experimentally confirmed the observation that E2 reduced miR-21 expression in MCF-7 cells. This repression was inhibited by the antiestrogen ICI 182,780 (Faslodex) and ERalpha knockdown by siRNA, indicating that the E2-suppression is ERalpha-mediated. E2 increased luciferase activity from reporters containing the miR-21 recognition elements from the 3'-UTRs of miR-21 target genes, corroborating that E2 represses miR-21 expression, resulting in a loss of target gene suppression. The E2-mediated decrease in miR-21 correlated with increased protein expression of endogenous miR-21-targets Pdcd4, PTEN, and Bcl-2. We are currently performing quantitative RT-PCR (Q-PCR) assessment of the miRNAs identified by microarray and examining changes in target protein expression by western blot analyses.

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212 Consistent Information From Cancer Expression Profiles Yields Agent-Specific Predictors of Chemotherapy Response

Qiyuan Li[1], Aron C. Eklund[1], Nicolai Juul[1], Christine Desmedt[2], Benjamin Haibe-Kains[2], Christos Sotiriou[2], W. Fraser Symmans[3], Lajos Pusztai[4], Andrea L. Richardson[5], **Zoltan Szallasi**[1,6]

[1]Center for Biological Sequence Analysis, Technical University of Denmark; [2]Medical Oncology Department, Jules Bordet Institute, Brussels, Belgium; Departments of [3]Pathology and [4]Breast Medical Oncology, University of Texas M. D. Anderson Cancer Center, Houston, TX; [5]Department of Pathology, Brigham and Women's Hospital, Boston, MA; [6]Children's Hospital Informatics Program, Harvard Medical School, Boston, MA

Genome scale expression profiling of human tumor samples is likely to yield improved cancer diagnosis. However, discovery of clinically predictive or prognostic classifiers has been hindered by the problem of overfitting, which results from a large number of noisy measurements taken in a small number of tumors. We describe an unsupervised method to extract robust, consistent metagenes from multiple analogous data sets. We applied this method to expression profiles from four triple negative (not expressing ER, progesterone receptor, or HER2) breast cancer cohorts and derived four metagenes. We applied these metagenes to four similar but independent cohorts and found a strong association between the metagenes and agent-specific response to neoadjuvant therapy. Furthermore, we applied the same approach to ovarian and early-stage lung cancer, two tumor types that lack reliable predictors of outcome, and found that the metagenes yielded predictors of survival for both.

213 Recombinant Human Erythropoietin Antagonizes Trastuzumab Treatment of Breast Cancer Via Janus Kinase 2-Mediated Activation of Src and Inactivation of PTEN

Ke Liang[1], Francisco Esteva[2], Constance Albarracin[3], Yang Lu[1], Yong Li[3], Xinqun Li[1], Chun-Te Chen[4], Ching-Yi Yang[2], Giampaolo Bianchini[2], Gordon B. Mills[5], Gabriel N. Hortobagyi[2], John Mendelsohn[1], Mien-Chie Hung[4], **Zhen Fan**[1,5]

Departments of [1]Experimental Therapeutics, [2]Breast Medical Oncology, [3]Pathology, [4]Molecular and Cellular Oncology, [5]Systems Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX

Recombinant human erythropoietin (rHuEPO) has been approved for preventing or alleviating cancer and cancer treatment-related anemia and fatigue since early 1990. Once thought to be only a hematopoietic cytokine, EPO is now known to be a pleiotropic cytokine characterized by remarkable cytoprotective activities in a variety of nonhematopoietic tissues, including neuronal, cardiac, retinal, and renal tissues, and more importantly in various cancerous tissues. The nonhematopoietic functions of EPO are biologically related to expression of EPO receptor (EpoR) recently discovered in these tissues.

Because EPO can activate a cascade of cell signaling via EpoR-associated Janus kinase-2 (Jak2) that largely overlaps with the signaling pathways activated by human epidermal growth factor receptor-2 (HER2), we hypothesized that concurrent rHuEPO treatment might play a role in conferring resistance to trastuzumab, an anti-HER2 antibody used to treat HER2-positive breast cancer patients. In a series of 55 cases of breast cancer specimens, we found that 13 out of 15 HER2-positive cases had various degrees of positive staining for EpoR. We demonstrated that concurrent treatment of HER2/EpoR dual-positive breast cancer cells with trastuzumab and rHuEPO reduced the response to trastuzumab both in culture and in a nude mice xenograft model. We found the underlying mechanisms involving Jak2-mediated activation of Src and inactivation of PTEN through which rHuEPO compensates trastuzumab-induced inhibition of cell signaling. Furthermore, we identified 1,941 women with breast cancer treated with rHuEPO at M. D. Anderson Cancer Center from 12/1998 to 02/2006; among those patients, 273 had received trastuzumab. We matched 50 metastatic breast cancer patients treated with first-line trastuzumab plus a taxane (with or without carboplatin) with another 50 metastatic breast cancer patients received comparable treatments but no concomitant rHuEPO. We found only the concomitant administration of rHuEPO was significantly correlated with a lower likelihood of objective clinical response. The correlation between rHuEPO administration and reduced response to first-line trastuzumab was significant after adjustment for age, estrogen receptor and/or progesterone receptor status, grade and carboplatin administration. Our results provide important preclinical and clinical evidence and mechanistic insights indicating that concurrent administration of rHuEPO and trastuzumab may be counteractive in patients with breast cancer that is positive for both EpoR and HER2.

214 Pharmacogenomically Selected Treatment for Gastric and Gastroesophageal Junction Tumors

A. Craig Lockhart[1], Laura W. Goff[2], Ben R. Tan[1], Howard L. McLeod[3], Richard M. Goldberg[3], Barbara A. Zehnbauer[1], Edith P. Mitchell[4], James A. Posey III[5], Yu Shyr[2], Wooin Lee[6]

[1]Washington University in St. Louis; [2]Vanderbilt University Medical Center; [3]University of North Carolina; [4]Thomas Jefferson University; [5]University of Alabama at Birmingham; [6]University of Kentucky

Background: Cancers of the gastric cardia and gastroesophageal junction (GEJ) have been rapidly increasing in incidence and unfortunately have a poor prognosis. Chemotherapy treatment selection for these patients remains largely empiric and in general treatment response rates and median survival have stagnated. Defining host and tumor characteristics in order to personalize treatment may improve outcomes in these patients. Germline polymorphisms in the number of tandem repeats in the TSER (thymidylate synthase enhancer region) have been shown to influence tumor TS mRNA transcription and translation efficiencies and potentially impact tumoral sensitivity to 5-FU. **Methods:** In this prospective, multi-institutional study, patients with advanced gastric or gastroesophageal junction cancers undergo pretreatment testing for TSER polymorphisms. Patients with at least one allele of germline TSER*2 polymorphism are expected to be sensitive to 5-FU and are selected to receive treatment with the FOLFOX-6 regimen every 2 weeks. We hypothesize that selecting treatment in this manner will result in a higher than historic value response rate of 60%. Host DNA as well as tumor tissue is being collected to retrospectively evaluate genomic and tumoral factors that may impact the outcome. **Results:** To date 23 patients have been consented to participate in the study at the four participating sites and 17 have been selected for treatment based on TSER genotyping. Ten patients have sufficient data for response rate assessment. By RECIST criteria, 5 patients have demonstrated a partial response (50%), 4 patients had stable disease (40%) and one patient had progressive disease (10%). Nine of 10 patients (90%) have demonstrated some degree of tumor shrinkage (range 2.5 to 60%). After 20 patients have been enrolled and are eligible for response assessment, an interim analysis will be performed to determine whether accrual should continue to the projected goal of 48 patients. **Conclusions:** This is the first multi-centered, prospective study of this kind in patients with gastric or GEJ cancer. Pretreatment genotyping for treatment selection is feasible in a multi-institutional study. Accrual to the study continues to determine whether TSER polymorphism status is an appropriate predictive marker for response to personalize chemotherapy treatment for patients with these cancers.

215 Development and Implementation of Genomic Predictors of Chemotherapy Response for Guiding Preoperative Therapy in a Prospective Breast Cancer Trial

P. Kelly Marcom, William Barry, Michael B. Datto, Joseph Geradts, Traci L. Foster, Laurie Rouse, Holly K. Dressman, John Olson Jr., Geoff Ginsburg, Anil Potti, H. Kim Lyerly, Joseph R. Nevins

Departments of Medicine, Biostatistics/Bioinformatics, Pathology, and Surgery, Institute of Genome Sciences and Policy, and the Comprehensive Cancer Center, Duke University Medical Center, Durham, NC

Genomic assays have been shown to provide the potential for personalized approaches to breast cancer therapy. Using an Affymetrix platform, we have developed genomic predictive signatures for various clinically relevant cancer characteristics. Previous credentialing studies have demonstrated signature sensitivity and specificity in retrospective studies. We report here on the investigation of the analytic performance of these signatures in biologic replicates, and their application in a prospective randomized clinical trial. **Methods:** To investigate the impact of intratumoral heterogeneity on the genomic signatures, patients with multiple frozen cores were identified in the Duke Breast SPORE tissue repository. Cores were assessed for percent invasive cancer cellularity and standard biomarker assessments. RNA was hybridized to H133 Plus 2.0 microarrays and gene expression signatures generated for previously identified predictors of sensitivity to adriamycin (A) and docetaxel (T). Genomic predictors of hormone receptor status were applied to post-processed array data, and compared to single-patient measures from IHC/FISH. The resulting analyses support the currently enrolling trial, "Performance of Genomic Expression Profiles to Direct the Use of Preoperative Chemotherapy for Early Stage Breast Cancer" a prospective randomized trial validating genomic signatures for predicting response to (A) or (T) treatment in HER2– cancers. **Results:** 51 samples from 18 patients were profiled to investigate the contribution of intratumoral heterogeneity to signature variation. The interclass correlation for the (A)– and (T)– sensitivity predictor in the replicate samples was 0.71 and 0.65 respectively ($p < 0.0001$). Expression data was also analyzed for a novel predictor of ER pathway activation. Predicted ER status among replicates showed perfect concordance using this pathway signature. Infrastructure has been established for conducting microarray analysis in support of the clinical trial, and provides microarray data in a clinically serviceable timeframe. Accrual to the study is underway. **Conclusions:** Microarray expression profiles are robust and reproducible, can be practically obtained and applied in the context of a prospective clinical trial, and can provide a variety of clinically useful prognostic and predictive signatures.

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216 Secondary BRCA1/2 Mutations Are Associated With Platinum Resistance in BRCA1/2-Mutated Ovarian Carcinomas

Barbara Norquist[1], Wataru Sakai[2], Kaitlyn Wurz[3], Beth Y. Karlan[4], Toshiyasu Taniguchi[2], Elizabeth M. Swisher[1]

[1]Division of Gynecologic Oncology, University of Washington, Seattle, WA; [2]Fred Hutchinson Cancer Research Center, Seattle, WA; [3]Department of Genome Sciences, University of Washington, Seattle, WA; [4]Cedars-Sinai Women's Cancer Research Institute, Los Angeles, CA

Background: BRCA1 and BRCA2 (BRCA1/2) proteins repair damaged DNA through homologous recombination. Primary carcinomas in BRCA1/2 mutation carriers are usually BRCA1/2-deficient because they have lost the wild-type BRCA1/2 allele. These tumors are initially sensitive to DNA cross-linking agents such as platinum compounds (cisplatin and carboplatin) but frequently develop acquired resistance to platinum. Secondary BRCA1/2 mutations that restore functional BRCA1/2 protein can be a mechanism of acquired platinum resistance, at least in a cell line model, and a small number of platinum-resistant ovarian carcinomas with secondary BRCA1/2 mutations have been reported. However, how frequently secondary BRCA1/2 mutations occur in BRCA1/2-mutated carcinomas or whether secondary BRCA1/2 mutations correlate with clinical platinum resistance has not been shown. **Objective:** We assessed clinical specimens of primary and recurrent BRCA1/2-mutated carcinomas for secondary mutations in BRCA1/2 and their correlation with platinum resistance and prior chemotherapy exposure. **Methods:** DNA was extracted from carcinomas occurring in BRCA1/2 mutation carriers after laser capture microdissection and sequenced at the site of the known germline BRCA1/2 mutation. When secondary mutations were found that restored wild-type sequence, haplotyping was performed using single nucleotide polymorphisms to rule out retention of the wild-type allele. Lymphocyte DNA was used for comparison. **Results:** Fifty-two primary and 35 recurrent ovarian carcinomas were assessed. Eleven of 35 recurrent carcinomas had secondary mutations, compared with 2/52 primary carcinomas ($p=0.0006$). Eight of 52 women with primary carcinomas had previous chemotherapy for breast cancer, and 2/8 had secondary mutations compared to 0/44 in women with no previous chemotherapy ($p=0.02$). Of recurrent carcinomas, 10/18 platinum-resistant cases had secondary mutations, compared with 1/15 platinum-sensitive carcinomas ($p=0.004$). Of seven other carcinomas assessed (six breast carcinomas and one rectal carcinoma), two had secondary mutations. Of 15 total secondary mutations, 13 were reversions that restored wild-type sequence, and two were new frameshift mutations that restored disrupted reading frame. **Conclusion:** Secondary mutations that restore functional BRCA1/2 protein occurred following chemotherapy and correlated with clinical platinum resistance. Future response to platinum can be predicted by the presence of secondary mutations in recurrent BRCA1/2 ovarian carcinoma.

217 Mechanisms of Sensitivity and Resistance to EGFR Tyrosine Kinase Inhibitors in Lung Cancer

William Pao

Vanderbilt-Ingram Cancer Center, Department of Medicine, Vanderbilt University, Nashville, TN USA

The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), gefitinib (Iressa) and erlotinib (Tarceva), induce dramatic responses in certain patients with non-small cell lung cancer (NSCLC). During the past few years, we have defined biomarkers that predict primary sensitivity, primary resistance, and secondary (or acquired) resistance to these drugs. Major predictors of primary sensitivity are EGFR kinase domain mutations, which predominantly comprise recurrent deletions in exon 19 and a recurrent point mutation in exon 21 (L858R). Markers of primary resistance include mutations in genes that encode signaling proteins downstream of EGFR (i.e., KRAS and BRAF). Patients with acquired resistance to these drugs develop other types of genetic alterations. Tumors in about one-half of such patients harbor second-site EGFR kinase domain mutations. The most common (>90%) second-site mutation involves a point mutation in exon 20 (T790M). Rarer mutations include D761Y and T854A. Another 20% of patients harbor tumors with amplification of the gene encoding another tyrosine kinase, MET. MET amplification occurs with or without T790M mutations. Transgenic mice with inducible expression in type II pneumocytes of EGFRT790M alone or together with a drug-sensitive EGFRL858R mutation develop lung adenocarcinomas that require mutant EGFR for tumor maintenance but are resistant to erlotinib. We are now using these animal lung tumor models to identify potential therapeutic strategies to overcome EGFRT790M-mediated resistance. For example, we have found that the combination of a new EGFR TKI, BIBW-2992, in conjunction with cetuximab, an anti-EGFR antibody, is synergistic and extremely effective at inducing complete tumor responses in mice bearing EGFRT790M-driven lung tumors. In primary mouse lung tumors, xenografts, and fibroblast transfectants, only the combination of both agents together induced near complete depletion of both phosphorylated and total EGFR. Novel dual targeting with cetuximab and a “second-generation” EGFR inhibitor may be an effective strategy to overcome T790M-mediated resistance.

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218 Biomarker Driven Clinical Trials by the Cancer and Leukemia Group B

Richard L. Schilsky

Cancer and Leukemia Group B, Chicago, IL

The Cancer and Leukemia Group B (CALGB) is a cooperative group sponsored by the National Cancer Institute (NCI) that conducts clinical trials, correlative science studies, and health outcomes research in all common adult solid tumors and hematological malignancies. The group is comprised of approximately 250 institutions and has 90–100 studies ongoing at any time that enroll about 3,500 patients annually. In addition to its headquarters in Chicago and Statistical Center at Duke University, CALGB maintains three biospecimen repositories, an imaging core laboratory, a pharmacology core laboratory, and three molecular reference laboratories that support the group's translational research activities. All biospecimens are obtained from patients enrolled on prospective clinical trials, and all are fully annotated with respect to patient demographics and outcomes. CALGB biorepositories are all compliant with NCI guidelines for biospecimen banks and contain collections of paraffin-embedded solid tumors, frozen lung cancer specimens with corresponding normal tissue, frozen leukemia cells, and germline DNA obtained for pharmacogenetic studies. CALGB conducts a variety of biomarker studies including retrospective-prospective studies, prospective drug-biomarker co-development studies, prospective biomarker validation studies, and prospective biomarker development studies. Ongoing and planned CALGB studies will screen for RAS mutation to determine eligibility for cetuximab-based treatment for colorectal cancer (C80405); assess FLT3 mutations in AML to determine patient eligibility for a study that assesses a novel FLT3 inhibitor (C10603); prospectively study the utility of celecoxib in NSCLC patients with COX-2 overexpressing tumors (C30801); use gene expression profiling to assign risk of relapse to patients with early stage NSCLC (C30506) or to determine whether treatment of those with late stage disease according to genome-guided drug selection improves patient outcomes (C30702). Studies in Hodgkin lymphoma and esophageal cancer will use risk-adapted treatment approaches based on the results of PET scans performed early in the course of treatment. In collaboration with other cooperative groups, CALGB is conducting studies to validate the utility of EGFR FISH testing to select therapy for patients with advanced NSCLC (MARVEL) and of the Oncotype Dx recurrence score to safely eliminate adjuvant chemotherapy in women with node negative, ER+ breast cancer and an intermediate risk score (TAILORx). The translational research studies of CALGB are transforming our approach to cancer treatment by enabling individualized treatment based on better understanding of cancer biology.

219 Activated Kinases and Signal Transducers in Melanomas

David F. Stern[1], Christina Zito[1], Kathryn Tworkoski[1], Antonella Bacchiocchi[2], Michael Krauthammer[1], Mario Sznol[3], Tisheeka Steed[1], **Ruth Halaban**[2]

Departments of [1]Pathology, [2]Dermatology, and [3]Oncology, Yale Cancer Center, Yale University School of Medicine, New Haven, CT

Clinical results suggest that treatments of melanomas and other cancers are likely to become more effective if guided by evidence-based test(s) that include some measure of an individual's tumor genotype, epigenotype, gene transcription, and/or pathway activation. This concept is particularly relevant to melanomas because they are highly heterogeneous with different etiological factors and molecular alterations. The findings that BRAF and c-KIT are mutated in melanoma provided the opportunity for targeted therapy with BRAF or c-KIT inhibitors, respectively. However, the observation that only a subset of patients respond to mutated and druggable targets suggests the need to invest major efforts in characterizing tumors with respects to other omics modalities to get a broad assessment of a patient's molecular profile that may confer resistance to therapy. Toward this goal, we performed genome-wide experiments, such as gene expression, SNP/CNV, and promoter methylation studies, as well as exon-sequencing. We recently added the high-throughput assessment of kinases and intracellular intermediates, analyzing the phosphorylation profiles of ~30 short-term cultures of freshly isolated melanoma cells employing the Proteome Profiler Antibody arrays of R&D Systems. This survey approach revealed frequently activated receptor kinases (RTK) and signal transducers in melanoma cell cultures. Several melanoma cell strains display multiple RTK activations, suggesting the need for combination therapies against these RTK targets. We are currently validating the capture-array data by Western blotting with motif-specific antibodies, investigating the causes of activation, testing the effect of BRAF inhibition of cells with different mutations, and performing bioinformatic analysis to cluster melanomas according to their pattern of kinases and intracellular pathway activation. These data, combined with the results of drug sensitivity assays, will enable us to develop systematic approaches for predicting responses to targeted drugs in melanoma therapy.

220 Validation of a Predictive Model of Clinical Response to Concurrent Radiochemotherapy in Patients With Locally-Advanced Esophageal or Rectal Cancer

Javier F. Torres-Roca, Steven Eschrich

H. Lee Moffitt Cancer Center and Research Institute

The ability to predict individual tumor radiosensitivity is central to the development of personalized treatment strategies in radiation oncology. Currently, there are no biomarkers that distinguish differences in intrinsic tumor radiosensitivity. Thus, the development of radiation-specific biomarkers can be a critical component for improving treatment using radiation therapy. In previous studies, we developed a systems biology model of intrinsic radiosensitivity in a 48 cell line database. The model identified a 10-gene system that we propose may serve as biomarker panel of intrinsic radiosensitivity. Using the 10 genes identified, we developed and optimized a linear regression algorithm to predict radiosensitivity. The algorithm predicts a radiosensitivity index (RSI) that is modeled on the survival fraction at 2 Gy, measured for the cell lines in the database.

We translated the cellular radiosensitivity model by applying the pre-defined 10-hub gene systems model to the prediction of clinical response to concurrent radiochemotherapy in two independent prospectively-collected pilot cohorts of patients with rectal (n=14) and esophageal cancer (n=12). All patients were treated with preoperative concurrent radiochemotherapy followed by surgery. Gene expression profiles were generated from pre-treatment biopsies. The model significantly separated responders (R) from non-responders (NR) in the clinical cohorts (all patients, mean predicted RSI, R vs. NR 0.34 vs. 0.48, p=0.002). Importantly, the model was accurate in both disease cohorts despite the small number of patients (rectal patients, mean predicted RSI, R vs. NR 0.32 vs. 0.46, p=0.03) (esophageal patients, mean predicted RSI, R vs. NR 0.37 vs. 0.50, p=0.05).

We further tested the model as a prognostic marker in 92 locally-advanced HNC patients treated with definitive concurrent radiochemotherapy (cisplatin-based) at the Netherlands Cancer Institute. Using the same algorithm described, we generated radiosensitivity predictions for this dataset. The predicted radiosensitive group had an improved 2-year locoregional control (2 year PFS 86% vs. 61%, p=0.05).

In summary, the systems model of cellular intrinsic radiosensitivity has been validated in three independent prospectively collected clinical cohorts totaling 118 patients in three different diseases. Therefore, we propose it is radiation-specific rather than disease-specific. Currently, the model is being prospectively tested in a clinical trial that will enroll 36 patients with esophageal or rectal cancer who are candidates for preoperative concurrent radiochemotherapy.

221 Genital Mucosal Immune Responses Are More Predictive of Lesion Regression in Preinvasive HPV Neoplasia Than Systemic Virus-Specific Response

Cornelia L. Trimble[1], Christopher Thoburn[1], Shiwen Peng[1], Nicole C. Hanson[3], Jodie Tassello[3] , Denise Frosina[3], Ferdynand Kos[1], Rachael A. Clark[2], Achim A. Jungbluth[3]

[1]Johns Hopkins University School of Medicine, Baltimore, MD; [2]Harvard Skin Disease Research Center, Department of Dermatology, Brigham and Womens' Hospital, Boston, MA; [3]Ludwig Institute for Cancer Research at Memorial Sloan-Kettering Cancer Center, New York, NY

Essentially all cervical squamous cell cancers and their precursor lesion, high grade cervical intraepithelial neoplasia (CIN2/3), are caused by persistent human papillomavirus (HPV) infection. Both are associated with viral integration into the host genome and subsequent expression of two viral oncoproteins, E6 and E7, which are functionally obligate for disease initiation and persistence. However, not all CIN2/3 lesions progress to cancer. In a brief, 15-week observational study protocol monitoring subjects from biopsy diagnosis (t0) to definitive therapy (cervical conization at twk15), we reported previously that 25% of CIN2/3 lesions associated with HPV16, the genotype most commonly associated with disease, underwent complete regression. HPV16-specific T cell responses measured in peripheral blood obtained at the time of study entry and at the time of conization were marginally detectable directly ex vivo and did not correlate with lesion regression. Here we present immunologic studies of the lesional mucosa: memory T cells accumulate in dysplastic mucosa, and spectratyping provides strong evidence that these populations often reflect clonal expansions. The degree of lesional intraepithelial CD8+ infiltration at the time of study entry was predictive of regression by week 15. In contrast, in lesions that failed to regress, immune cell infiltrates increased in individual subjects but were restricted to the stromal compartment, while intraepithelial CD8+ infiltrates remained minimal. Mechanisms by which preinvasive HPV-associated epithelial lesions are likely to mediate immune evasion well before development of an invasive phenotype will be discussed.

222 Genetic Variations Predict Clinical Outcomes of NSCLC Patients

Xifeng Wu[1], Jack A. Roth[1], Yuanqing Ye[1], David J. Stewart[1], Charles Lu[1], Michelle A.T. Hildebrandt[1], Jian Gu[1], Jie Lin[1], Jessica Clague[1], Joe Y. Chang[1], Ritsuko Komaki[1], Scott M. Lippman[1], Waun Ki Hong[1], Margaret R. Spitz[1], John D. Minna[2], Ping Yang[3]

[1]The University of Texas M. D. Anderson Cancer Center; [2]University of Texas Southwestern Medical Center; [3]Mayo Clinic College of Medicine

Inter-individual variation in genetic background may influence clinical outcomes of non-small cell lung cancer (NSCLC) patients. We have taken several approaches, including comprehensive pathway-based and genome-wide scan studies, to identify common genetic polymorphisms associated with toxicity and survival in advanced NSCLC patients. For NSCLC patients treated with radiation, the risk of developing severe radiation-induced toxicity is high and often results in suboptimal treatment. We utilized a pathway-based approach to evaluate 59 SNPs from 37 inflammation-related genes and identified several variants that modulated an individual's susceptibility to developing either esophagitis or pneumonitis. There was a gene-dosage effect, as evidenced by a significantly increased risk of toxicity with an increasing number of risk genotypes. The Sonic Hedgehog signaling pathway has been shown to be deregulated in many cancers, resulting in cancer progression. We hypothesized that genetic variation within this pathway may alter NSCLC patients' prognosis. To test this, we genotyped 154 SNPs among eight Sonic Hedgehog pathway genes and evaluated their associations with overall survival. Several associations were identified, particularly in the GLI transcription factor genes. Survival tree analysis revealed potential higher-order gene-gene interactions among these variants that further affect overall survival. Finally, we conducted a genome-wide scan of 327 NSCLC patients followed by a fast-track replication with an additional 315 patients in collaboration with the Mayo Clinic. A SNP (rs10937823) located in an intron of the sortilin-related VPS10 domain containing receptor 2 (SORCS2) gene was significantly associated with overall survival in both discovery and validation sets. The combined analysis showed that the variant allele containing genotype conferred a 1.82-fold increased risk (95% CI: 1.42 – 2.33, P = 1.73E6) and conferred a significant survival advantage of 5 months. Overall, using multiple approaches, these studies have identified several new candidate biomarkers for NSCLC response to therapy and overall survival. Together with known epidemiologic, clinical, and genetic markers, we can enhance the identification of patients who will have a poor prognosis and response to therapy.

223 Genetic Variations in SLCO2B1 and SLCO1B3 Are Associated With the Efficacy of Androgen Deprivation Therapy in Prostate Cancer Patients

Ming Yang, Wanling Xie, Elahe Mostaghel, Tong Sun, Nima Sharifi, Meredith Regan, Mark Pomerantz, Matthew Freedman, Robert Ross, William D. Figg, Steven Balk, Myles Brown, William Oh, Gwo-Shu Mary Lee, **Philip Kantoff**

Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA; Cancer Biology Program, Department of Medicine, Division of Hematology Oncology, University of Washington; Division of Hematology/Oncology University of Texas Southwestern Medical Center; Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA; Molecular Pharmacology Section, National Cancer Institute, NIH, Bethesda, MD

At least two members of the organic anion-transporter gene family, SLCO2B1 and SLCO1B3, are involved in the androgen metabolic pathway. SLCO2B1 transports dehydroepiandrosterone sulfate (DHEAS), an androgen precursor that is responsible for as much as one-half of the androgen production in prostate tissue. SLCO1B3 is involved in testosterone uptake. Therefore, we hypothesized that the differential uptake of DHEAS or testosterone by SLCO2B1 or SLCO1B3 variants in prostate cancer cells could result in differences in time to progression (TTP) during androgen-deprivation therapy (ADT). A cohort of 538 men with advanced prostate cancer treated with ADT was genotyped for 20 SNPs in SLCO2B1 and 2 previously identified missense SNPs in SLCO1B3. Three polymorphisms in SLCO2B1 (rs12422149A>G [Arg312Gln], rs1789693A>T and rs1077858A>G) were significantly associated with TTP during ADT in multivariate analyses ($P < 0.05$). There was also an additive effect of combinations of genotypes across the three loci. In LNCaP cells, we demonstrated that the SLCO2B1-312Gln and SLCO2B1-312Arg variants in LNCaP cells differentially transport DHEAS, with the 312Gln variant showing consistently higher DHEAS uptake ability than the 312Arg variant. These functional data are consistent with and complement the observed clinical associations with TTP. In addition, a gene-gene interaction between SLCO2B1 and SLCO1B3 SNPs and TTP was observed in that people carrying the more efficient DHEAS transporting SLCO2B1 alleles and more efficient transporting testosterone SCCO1B3 alleles demonstrated a shorter TTP. In conclusion, these results highlight that genetic variants in SLCO2B1 may influence the efficacy of ADT, separately or in combination with SLCO1B3 polymorphisms, and may provide mechanistic insight into ADT responsiveness and resistance.

[*Table presented on the following page*]

No. of good genotypes in SLCO2B1	N	Median (mos)	HR (95% CI)	P value
All patients				
0	50	12.8	1.00(ref)	<0.0001*
1	204	19.0	0.82 (0.58,1.16)	
≥2	235	30.9	0.52 (0.37,0.74)	
By SLCO1B3 genotype				
rs4149117=GG				
0	29	14.0	1(reference)	
1	139	21.1	0.76(0.47,1.23)	
≥2	167	28.1	0.63(0.39,1.01)	
rs4149117=GT/TT				
0	20	11.1	1(reference)	
1	64	15.5	0.67(0.40,1.12)	
≥2	67	37.4	0.28(0.16,0.49)	0.041**

log rank p

**Wald Chi-square test for interaction from Cox regression

224 Pharmacogenetic Profiling in Angiogenesis Pathway Predict Clinical Outcome in NSCLC Patients Treated With Bevacizumab in Combination With Carboplatin and Paclitaxel: Pharmacogenetic Analysis of E4599

Wu Zhang[1], Suzanne E. Dahlberg[2], Dongyun Yang[1], Alan B. Sandler[3], Julie R. Brahmer[4], Joan H. Schiller[5], David P. Carbone[3], David H. Johnson[3], Afshin Dowlati[6], **Heinz-Josef Lenz**[1]

[1]University of Southern California/Norris Comprehensive Cancer Center, Keck School of Medicine, Los Angeles, CA; [2]Dana-Farber Cancer Institute, Boston, MA; [3]Vanderbilt Ingram Cancer Center, Nashville, TN; [4]SKCCC at Johns Hopkins, Baltimore, MD; [5]University of Texas Southwestern, Dallas, TX; [6]Case Western Reserve University, Cleveland, OH

Background: E4599 was a randomized phase III study that demonstrated a survival advantage in advanced NSCLC patients treated with bevacizumab (bev) + carboplatin/paclitaxel (BPC) versus carboplatin/paclitaxel alone (PC). A recent study has shown germline polymorphisms involved in VEGF pathway were associated with clinical outcome in advanced breast cancer patients treated with paclitaxel plus bevacizumab. Frey et al. also found 2 SNPs in WNK1 gene were associated with bevacizumab-induced hypertension. We tested the hypothesis that polymorphisms involved in angiogenesis pathway (VEGF, EGF, EGFR, IL-8, KDR, ICAM1, and FGFR4), DNA repair pathway (ERCC1, XPD, XRCC1, GSTP1), and WNK1 will predict clinical outcome and toxicity in a subset of patients enrolled on E4599.

Patients and method: A total of 878 patients enrolled in E4599, and samples from 146 patients were available for the current pharmacogenetic study. One hundred thirty-three patients (67 from PC arm, 66 from PCB arm) are eligible for correlative analysis. PCR-RFLP assays were performed on genomic DNA extracted from sera of patients using QIAamp DNA extraction kit.

Results: Median OS for the 133 patients was 10.3 months (8.2–15.6) for PC arm and 13.0 months (10.2–16.6) for PCB arm. Median PFS was 4.6 months (3.6–5.6) for PC arm and 6.5 months (5.4–8.3) for PCB arm. Patients with mutant homozygote CC genotype for ICAM1 T469C had significantly higher tumor response rate (39%) than heterozygote CT (13%) and homozygote TT (20%) genotype (Fisher's test, p=0.04). Tests for whether treatment effect differs by genotype via interaction terms in the Cox models were statistically significant (p<0.05) for OS and PFS by selecting a panel of polymorphisms including VEGF G-634C, ICAM1 T469C, and IL-8 T-251A.

Conclusion: Although exploratory, our preliminary results suggest germline polymorphisms in angiogenesis pathway may predict response, PFS and OS in NSCLC patients treated with bevacizumab-containing regimen. Prospective trials based on these correlative studies are warranted.

225 p53 Autoantibodies as Potential Detection and Prognostic Biomarkers in Serous Ovarian Cancer

Karen S. Anderson[1,5], Jessica Wong[1], Chris Crum[2], Allison Vitonis[2], Patrick Sluss[3], Joshua LaBaer[4], Daniel Cramer[2,5]

[1]Cancer Vaccine Center, Dana-Farber Cancer Institute, Boston, MA; [2]Department of Gynecology and Reproductive Biology, Brigham and Women's Hospital, Boston, MA; [3]Department of Pathology, Massachusetts General Hospital, Boston, MA; [4]Center for Personalized Diagnostics, Biodesign Institute, Arizona State University, Tempe, AZ; [5]Department of Medicine, Harvard Medical School, Boston, MA

Purpose: This study sought to examine the specific value of serum p53 autoantibodies (p53-Abs) as detection and prognostic biomarkers in ovarian cancer.

Experimental Design: A matched case-control cohort of sera (n=60) were obtained pre-operatively from women with serous ovarian cancer or from age-matched healthy women (n=60) selected from the general population. Sera were randomly divided into independent training sets, non-serous carcinomas, and blinded validations sets (n=30 cases/30 controls each). A custom RAPID ELISA assay for p53-Abs, using reticulocyte lysate to express recombinant p53-GST fusion protein, was used to detect p53-Abs in the sera. Clinical correlation with ovarian cancer risk factors, p53 antigen expression, immune response polymorphisms, CA125 and HE4 levels, and overall survival were determined in multivariate analysis.

Results: p53 IgG Abs were specifically detected at high titer (1:4,600-1:48,600) in ovarian cancer patient sera. In the training set of serous carcinomas, p53-Abs were detected in 13/30 (43.3%) patient sera and 0/30 (0%) of control sera. In the independent blinded validation set, p53-Abs were detected in 12/30 (40.0%) of patient sera and 1/30 (3.3%) of control sera (combined $p<0.0001$). p53-Abs were not specifically detected in sera from patients with non-serous cancers (n=30). In this cohort, p53-Abs did not significantly improve detection of cases (AUC=0.69) or discrimination of benign disease (AUC=0.64) compared with CA125 (AUC=0.99) or HE4 (AUC=0.98). The presence of p53-Abs in the sera was independent of age, oral contraceptive use, and parity. In multivariate analysis, p53-Abs correlated only with a family history of breast cancer ($p=0.01$) and were independent of CA125 and HE4 levels. Detectable p53 antibodies in pre-treatment sera correlated with improved overall survival ($p=0.06$).

Conclusions: Antibodies to p53 are detected in the sera of 41% of patients with advanced serous ovarian cancer at the time of diagnosis and are prognostic for improved overall survival.

226 Microenvironmental Factors Predict Prostate Cancer Specific Death

Gustavo Ayala, Anna Frolov, Rile Li, Michael Ittmann, Peter Scardino, Thomas Wheeler, David Rowley

Baylor College of Medicine

Most cancers have a survival curve that plateaus after 5 years, as most disease-specific deaths occur within this period. In contrast, PCa-specific death continues to occur up to the 20th year of followup. BCM currently has a retrospective cohort with 20 years of followup, tissues that are adequate for analysis and full clinicopathologic characterization. The TMA was immunostained and slides were digitized and quantified using an expression index (Intensity*Percentage) for nuclear and cytoplasmic expression and in some cases the activated (phosphorylated) versus the inactive from of the protein. Biomarkers included: proliferation (Ki-67) and apoptosis (TUNNEL and Caspase-3), survival pathways (AKT, SKP-2, GSK, FHKR, p27, PTEN, PIM2, C-MYC, FRAT, PKC and NFkB), hormonal regulation (DAX1, AR, ER, and SRC 1, 2, and 3). Stroma was represented through RSG, stromal caveolin, FGF4, and ps20. Other biomarkers included RTVP VEGFR3, ASCT2, RTVP, HAIN, B-CAT, REG Gamma, SPINK, NCoR and Sprouty. Ki67 and TUNEL were counted as number of positive cells/100 cells. A total of 236 variables were examined. Clinico-pathologic parameters that were significant on univariate analysis were clinical staging (UICC), lymph node status (LN), extracapsular extension (ECE), seminal vesicle invasion (SVI), margins, and Gleason. A multivariate model of clinicopathologic parameters alone demonstrated that only SVI (HR=5.36, p=<0.001) and GGTOT (HR=2.73, p=<0.001) remained significant predictors of Pca-specific death. Subsequently, we analyzed the predictive value of the biomarkers in our database. On univariate analysis SKP2, caspase 3, P-AKT in BPH, P-AKT in non neoplastic tissues, tumor volume, tumor size, RSG3 volume, nuclear REG gamma, desmin in reactive stroma, PNI diameter, KI67 and nuclear PIM2 in PNI cells were predictive. Of note is the presence of biomarkers of normal tissue such as P-AKT and TME biomarkers such as RSG and PNI diameter. We have noted repeatedly in a number of previous studies that biomarkers of normal tissues hold predictive information. Finally, all significant variables were introduced into the model. Only 3 biomarkers were independently predictive of Pca-specific death (REG gamma, % of RSG 3 and PNI diameter). This data supports our hypothesis that the TME holds significant predictive information for both biochemical recurrence as well as Pca-specific death and represents a new paradigm in tumor biomarker research.

227 Predictive and Prognostic Molecular Markers of Colorectal Cancer of African-American Patients

Harvey Bumpers[1], Upender Manne[2]

[1]Morehouse School of Medicine; [2]University of Alabama School of Medicine at Birmingham

Colorectal cancer (CRC) accounts for the death of 55,000 Americans annually. 5-Fluorouracil (5FU) remains the most common chemotherapeutic agent used in CRC treatment and accurate predictive factors of 5FU-based therapy are needed. In preliminary studies, therapeutic efficacy may significantly differ based on genetic and molecular determinants associated with race/ethnicity. We hypothesize that the ability of phenotypic and molecular factors to predict the efficacy of chemotherapy and prognosis will vary with anatomic location, tumor stage, and race. To determine the predictive value of aberrant phenotypic expression, we collected sporadic Stage II and III CRCs from different locations in the colon of African-American (AA) patients. They had adjuvant treatment with 5FU/leucovorin or 5FU/leucovorin/oxaliplatin (FOLFOX) or surgery alone. Archived AA specimens (1990-2005) were collected from University of Alabama at Birmingham (UAB) and Morehouse School of Medicine (MSM). Since the anti-tumor activity of 5-FU based therapy induces apoptosis, we are evaluating IHC expression of Bax, Bcl-2 and p53 (involved in apoptosis); TS, TP, and DPD (metabolism of 5FU); p21waf-1, p27kip-1 and Ki67 (cell cycle markers); and EGFR, VEGF, and MUC1 (therapeutic targets) in primary sporadic CRCs. The DNA extracted is assessed for microsatellite instability (MSI, a potential prognostic/predictive marker). Our studies evaluate tissues collected from 1,600 AA (800 from UAB and 800 from MSM). MSI status will be compared between responders and non-responders to 5-FU therapy as well as correlated with time to recurrence and survival based on tumor location. This full project of NIH/NCI-funded U54 grant (CA118948-UAB/MSM/Tuskegee partnership) was initiated in 2008, and a total of 1,174 (535 from UAB and 639 from MSM) cases have been screened for demographics, treatments, pathologic features, and tumor location. Anonymous identity codes were assigned for all specimens. The interim molecular data obtained for 256 samples demonstrate that increased expression of Bcl-2 in cancers has a positive prognostic and predictive value. CRCs that exhibited stable MS had bad prognosis but a better response rate to 5-FU based therapy. Preliminary findings suggested that a single nucleotide polymorphism at codon 72 of the p53 gene was common in AA patients, and specifically the proline/proline phenotype of this SNP demonstrated aggressive tumor progression resulting in early CRC-specific deaths in AA patients.

228 Biological Pathways That Mediate Drug Resistance in Childhood Acute Lymphoblastic Leukemia (ALL)

William L. Carroll

NYU Cancer Institute, NYU-Langone Medical Center, New York, NY

The prognosis for children with ALL has improved dramatically over the past four decades with cure rates now exceeding 80%. However, the prognosis is dismal for patients who suffer a recurrence. Multiple attempts to improve outcome using intensive chemotherapy, including the use of stem cell transplantation, have failed. We have used high throughput genomic techniques to discover the underlying biological pathways associated with relapse by analyzing a cohort of diagnosis/relapse sample pairs from children enrolled on Children's Oncology Group Protocols. To date, we have analyzed gene expression profiles (GEP) in 60 pairs (120 samples, Affymetrix U133A and U133 Plus 2.0) as well as copy number abnormalities (CNAs) in 50 pairs (150 samples that included germline samples, Affymetrix 500K SNP). Relapsed ALL is characterized by over-expression of genes involved in cell proliferation, cell cycle control and DNA repair (e.g., BIRC5, PTTG1, RAD51) and downregulation of genes involved in mediating apoptosis (e.g. p21, TNFPAI3, BCLAF1). Interestingly, there were distinct signatures that differentiated early from late relapse. Upregulation of genes involved in mitosis, and cell cycle regulation dominate early relapse cases, whereas upregulation of genes involved in nucleotide metabolic processes were seen in late relapse blasts. It is noteworthy that genes whose proteins products are targets for maintenance chemotherapy (DHFR, TYMS) are upregulated in blasts from patients who suffer a later recurrence. In a pilot cohort of 20 diagnosis and relapse leukemia pairs, we noted a number of CNA that varied significantly among patients, ranging from 3 to 84 per sample. The median size of CNAs identified was 353 Kb, with 22.7% < 100Kb, and 66.4% < 1 Mb. The median copy number loss per sample was 9 at diagnosis and 9.5 at relapse. Copy number gains were less common ($P<0.001$, 3.5 events per sample at diagnosis and 4 at relapse). There was a modest increase of CNAs at relapse ($P=0.035$). Twenty-four novel amplifications and 45 novel deletions arose at relapse, accounting for 25.0% and 14.9% of total copy number gains and losses at relapse, respectively. In the expanded set of 50 pairs we have observed relapse specific CNAs including deletions of MSH6 (3 cases), IKZF1 (2 cases), EBF1 (2 cases), CyclinG2 (2 cases) NR3C1 (2 cases) and amplifications of ARHGAP22 (4 cases), CKSIB (5 cases), and CXCL12 (2 cases) among others. These studies provide insight into mechanisms associated with tumor recurrence, and efforts have already validated some of these targets in preclinical models that will serve as the basis for clinical trials.

229 Molecular Diagnois and Target Directed Therapy in Non-Hodgkin Lymphoma

Wing C. Chan[1], Randy D. Gascoyn [2], Elias Campo[3], Konrad Mueller-Hemerlink[4], Liza Rimza[5], Erlend Smeland[6], Andrew Liste[7], Louis M. Staud[8]

Lymphoma/Leukemia Molecular Profiling Project LLMPP: [1]University of Nebraska Medical Center; [2]British Columbia Cancer Agency; [3]University of Barcelona; [4]University of Wurzburg; [5]Southwest Oncology Group, University of Arizona, University of Rochester, University of Oregon, Cleveland Clinics; [6]Radium Hospital, Norway; [7]St. Bart, London, UK; [8]National Cancer Institute

Non-Hodgkin Lymphoma (NHL) is a very heterogeneous group of disorder and even within a single diagnostic entity, there is significant heterogeneity in survival indicating divergent activation of oncogenic pathways. We have studied over 2,000 NHL cases using global approaches such as gene expression profiling (GEP) and array comparative genomic hybridization (aCGH). The goal is to provide an accurate diagnosis for each lymphoma at presentation including key abnormal oncogenic pathways so that therapy may be individualized.

In the most common type of NHL, the diffuse large B-cell lymphoma (DLBCL), we have identified 3 major subtypes, the germinal center B-cell like (GCB), the activated B-cell Like (ABC), and the primary mediastinal (PM)-DLBCL. They differ in their GE profiles, pattern of genetic abnormalities, and clinical outcome. Notably, the NF-KB pathway tends to be highly active in the ABC-DLBCL. We and others have identified mutations of TNFAIP3, CARD11, TNFSF11A and other members as one of the mechanisms of NF-KB activation. Interestingly, gain/amplification affecting the microRNA cluster Mir17-92, occurs exclusive in the GBC-DLBCL. Mir17-92 down-modulate PTEN and PHLPP2 expression leading to enhanced AKT-1 phosphorylation that synergizes with BIM1 downregulation in antagonizing apoptosis. We have attempted to reproduce the DLBCL classification in paraffin embedded materials using an immunohistochemical or a microRNA platform and have obtained over 90% concordance with either.

In follicular lymphoma, we have demonstrated the prominent influence of tumor/host interaction on survival. In contrast, in mantle cell lymphoma, the proliferation signature, which may reflect the convergence of factors dysregulating the cell cycle such as aberrant cyclin-D1 expression and loss of p16/ARF exerted control, is the dominant prognosticator.

These genome scale investigations have helped to unravel multiple oncogenic pathways in the different NHLs. They will continue to provide fresh insight in the future, especially with the addition of high throughput sequencing that is particularly relevant in B-NHL as many of which have aberrant ongoing somatic hypermutation and class switch recombination.

230 Assessing Prostate Cancer Growth With mRNA of Spermine Anabolic Enzymes

Leo L. Cheng

Massachusetts General Hospital, Harvard Medical School

Background: Clinical data indicate that a large portion of prostate cancer discovered by prostate specific antigen screening may be latent disease. However, current medical tests cannot differentiate slow- from fast-growing tumors in many of these patients, resulting in many unnecessary radical treatments causing morbidity.

Methods: Inspired by the reported studies of inhibitory function of spermine in the growth of prostate cancer cells, we investigated spermine and mRNA expression levels of rate-limiting enzymes in the spermine biosynthesis pathway. Using spectroscopic, histopathological, laser capture microdissection and real-time PCR techniques, we observed correlations between spermine and mRNA expression levels and human prostate cancer growth rates represented by serum prostate specific antigen velocity.

Results: We observed that the expression levels of spermine anabolic enzymes ODC and AdoMetDC in benign epithelia surrounding cancer glands (El) was logarithmically reduced with the increase of Vpsa (ODC, $p< 0.016$; AdoMetDC, $p< p<0.048$) and that antizyme (OAZ) expression in cancer cells increases with the increase of PCa growth rate represented by Vpsa ($p< 0.001$). Finally, inverse correlations observed locally in cancer cells between ODC and OAZ ($p< 0.019$) and between AdoMetDC and oncogene c-MYC ($p< 0.017$) agree with the mechanisms that malignancy developments increase the expressions of c-MYC and OAZ and reduce ODC and AdoMetDC expressions.

Conclusions: These correlations can function to evaluate the aggressiveness of human prostate cancer and assist patients and clinicians with selecting appropriate treatment strategies based on biological activities of individual tumors.

231 A Predictive Model Incorporating Biomarkers for Outcome in Malignant Pleural Mesothelioma

Assunta DeRienzo[1], Lingsheng Dong[1], Beow Y. Yeap[2], Gavin J. Gordon[1], William G. Richards[1], David J. Sugarbaker[1], **Raphael Bueno**[1]

[1]Division of Thoracic Surgery, Brigham and Women's Hospital and Harvard Medical School, Boston, MA; [2]Department of Medicine (B.Y.Y.) Massachusetts General Hospital and Harvard Medical School, Boston, MA

Background: Malignant pleural mesothelioma (MPM) is a rapidly fatal neoplasm with few effective treatments, one being cytoreductive surgery. We previously described a predictive test, based on ratios of gene expression levels, for the outcome of MPM patients undergoing surgery. Here we incorporated this test with additional prognostic parameters to develop a new staging paradigm and extended it to fine needle aspiration (FNA) biopsies. **Methods:** Tumor specimens linked to clinical data were prospectively obtained from 120 consecutive patients undergoing surgery for MPM. FNAs were obtained from the same specimens. A 4-gene predictive test was used to assign patients to either a good- or poor-outcome group. This test was evaluated in multivariate modeling to assess its predictive value independently and in combination with other proposed prognostic variables. The accuracy of FNA analysis of the predictive test was also evaluated in a subset of the specimens by comparing the same test results utilizing specimens obtained as tissue biopsies and FNAs. **Results:** The test predicted overall survival and cancer specific survival in a statistically significant manner in multivariate analyses. When combined with other known clinical prognostic variables such as lymph node status, tumor volume, and histologic subtype, each factor was an independent statistically significant variable in a multivariate model. A predictive scheme combining all four variables was developed that assigned patients into three subgroups with the rates of overall survival at 3 years being 42%, 12%, and 0%, respectively. The predictive tests performed on FNAs were highly correlated with those performed on tissue biopsies. **Conclusions:** The MPM predictive test can be combined with other prognostic parameters to accurately predict patient outcome and define which patients might benefit from aggressive surgical approaches. All of these predictive parameters can be obtained and determined prior to major surgery, enabling a personalized selection of treatment options. Real-time RTPCR analysis of FNA specimens for the MPM predictive test can be used to reduce any morbidity associated with tumor biopsies.

232 Breast Cancer Molecular Profiles Are Predictive of Tumor Response to Neoadjuvant Chemotherapy: The I-SPY TRIAL (ACRIN 6657, CALGB 150007/150012, InterSPORE)

Laura J. Esserman[1], Charles M. Perou[2], Maggie Cheang[2], Angela DeMichele[3], Lisa Carey[2], Laura J. van 't Veer[4], Joe Gray[1], Kathleen Conway[2], Baljit Singh[5], Meredith Buxton[1], Sarah E. Davis[1], Donald Berry[6], Nola Hylton[1], and the I-SPY Investigators

[1]University of California, San Francisco, San Francisco, CA; [2]University of North Carolina at Chapel Hill, Chapel Hill, NC; [3]University of Pennsylvania, Pittsburgh, PA; [4]Netherlands Cancer Institute, Amsterdam, Netherlands; [5]New York University, New York, NY; [6]M. D. Anderson Cancer Center, Houston, TX

The I-SPY TRIAL is a neoadjuvant multi-center trial designed to identify predictive markers of pathological complete response (pCR) and survival of women with locally advanced breast cancers (>/=3cm). Patients were treated with AC->T. **Methods:** 237 patients enrolled; 216 completed serial imaging and core biopsies. Pre-treatment assays included Agilent expression arrays, MIP aCGH, p53 gene chip and sequencing, and IHC. Response to therapy was measured by serial MRI, pCR and residual cancer burden (RCB), a quantitative histopathologic measure of cancer that identifies a group near pCR (RCB I). Associations among molecular markers, pCR, RCB and survival were evaluated using chi-square tests, K-M curves, and log-rank test. All data are centrally stored on caINTEGRATOR. **Results:** Median tumor size was 6cm, with 27% pCR and 36% RCB 0/I; 25% pCR for the 144 Agilent arrays. Several molecular subtypes, including NKI 70 gene low, luminal A, and IHC Hormone Receptor+ (HR), define 9–48% of patients with 0–10% pCR, yet excellent early survival; whereas patients with high risk molecular profiles: NKI 70 gene high (91%), IHC HR- Her2+ (12%) or IHC HR-Her2- (28%), activated wound healing (77%), and basal subtype (32%) define patients with pCR rates of 28-59% to standard chemotherapy. RCB was more predictive of DFS & OS ($p=0.01$) than pCR alone with a mean follow up of 3.9 years. MR volume is highly predictive of pCR and RCB. For poor risk subtypes, RCB is incredibly predictive of DFS ($p<0.001$). **Conclusion:** Patients with good prognosis profiles had few or no recurrences at 3 years in spite of low rates of response to chemotherapy. Yet patients with high-risk profiles had a good response to chemotherapy, as measured by RCB, is incredibly predictive of recurrence at 3 years. I-SPY TRIAL data support the need to target improvement to pCR/RCB to improve outcomes in poor prognosis patients and provides a platform to compare, contrast, and combine marker signatures to tailor therapy. This is the foundation of the I-SPY 2 TRIAL.

I-SPY 2, a collaboration of NCI, FDA, and fNIH BC, is a neoadjuvant phase 2 adaptive design trial process to test novel agents in combination with chemotherapy to improve the rate of pCR in women with poor risk profiles. I-SPY 2 will extend the IT infrastructure from I-SPY 1, and eliminate many of the inherent organizational ineffeciencies in clinical trials. Predicted likelihood of success in phase 3 trial & predictive biomarkers will accompany each drug that leaves the trial. I-SPY 2 will be a model for accelerating the pace of identifying and developing molecularly targeted agents and improving outcomes for women with high risk cancers.

233 Evaluation of the Effect of Screening on the Detection of Good and Poor Prognosis Breast Cancers

Yiwey Shieh[1], Laura van't Veer[2], Sabine Linn[2], Emiel J.T. Rutgers[2], Michael Knauer[2], Valesca Retel[2], Sarah E. Davis[1], **Laura J. Esserman**[1]

[1]University of California, San Francisco; [2]Netherlands Cancer Institute (NKI)

Introduction: It is being increasingly recognized that the incidence of breast cancer may be related to the diagnosis of low risk tumors that would not have otherwise come to clinical attention. The bulk of the rise in the United States has been observed in women over 50 years old, with minimal change in the incidence of women below 40. We are seeking to use molecular profiles to identify the fraction of biologically low-risk tumors that are being detected with screening. In addition, we are seeking to define an "ultralow" risk group of patients to determine if such a profile might be applied to help mitigate the potential overdiagnosis as a consequence of mammography and subsequently avoid local and systemic overtreatment for such cancers. **Methods:** Results of the 70-gene prognostic signature test developed by van't Veer et. al (Mammaprint) were compiled from two previously published studies. The first, the European Validation Study (EVS), includes 302 patients diagnosed with Stage I or II cancers between 1980–98. The second, RASTER, includes 427 patients treated at community hospitals in the Netherlands who were recruited to a prospective trial between 2004–6. The screening attendance of participants was ascertained from study records (RASTER) or estimated according to historical data on the implementation of screening (EVS). The proportions of Mammaprint high-risk versus low-risk cancers were compared across the two studies. Low-risk signatures were defined and validated as having a 5-year metastasis-free survival (MFS) of >90%. Ultralow tumors are a subset of low-risk cancers whose signatures were associated with 5-year MFS of almost 100%. **Results:** The EVS included 302 patients from age 28–60, Stage 1 and 2, LNneg, adjuvantly untreated; it represents a largely unscreened cohort. Within women aged 50–60 in the EVS, 38% had a low-risk and 62% had a high-risk signature. The RASTER dataset included 427 patients, LNneg, from age 27–60, for whom no selection based on subsequent treatment was made; it represents a modern screened cohort. Within women aged 50–60 in RASTER, 61% had a low-risk and 39% had a high-risk profile: 70% were Stage 1 and 29% were Stage 2. For women aged 40 and under, the distribution was 30% low-risk and 70% high-risk in both datasets. Results for ultralow risk cancers are being determined and will be available by the end of August 2009. **Conclusion:** The results suggest that a significant increase in good prognosis tumors has resulted from nationwide screening programs. Molecular characterization may help reduce overtreatment and enable exploration of less aggressive treatment approaches for low and ultralow risk tumors.

234 Hypoxia-Inducible Factor-1 Alpha (HIF-1a) Expression Predicts Superior Survival in Diffuse Large B-Cell Lymphoma Patients Treated With R-CHOP

Andrew M. Evens[1,2], Laurie H. Sehn[3], Pedro Farinha[4], Adekunle Raji[2,5] Yi Lu[5], Jane N. Winter[1,2], Paul T. Schumacker[2,6], Randy D. Gascoyne[4], Leo I. Gordon[1,2]

[1]Division of Hematology/Oncology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL; [2]The Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago, IL; Departments of [3]Medical Oncology and [4]Pathology, British Columbia Cancer Agency (BCCA), Vancouver, BC, Canada; Departments of [5]Pathology and [6]Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, IL

Purpose: Hypoxia-inducible factor (HIF) controls the expression of genes in response to hypoxia as well as a wide range of other cellular processes. We previously showed constitutive stabilization of HIF-1-alpha (HIF-1a) in the majority of diffuse large B-cell lymphoma (DLBCL) cases. The prognostic significance of HIF in lymphoma has never been investigated.

Methods: We studied the immunohistochemical protein expression of HIF-1a on tissue microarrays from 153 DLBCL patients treated in sequential cohorts with CHOP or rituximab-CHOP (R-CHOP) from 1999-2002. Results were correlated with patient outcome.

Results: Median followup for all patients was 80 months. Among all patients, HIF-1a was expressed in 62% of germinal center and 59% of non-germinal center patients. With HIF-1a analyzed as a dependent variable, there were no survival differences in CHOP-treated patients. In the R-CHOP group, however, HIF-1a protein expression correlated with significantly improved progression-free survival (PFS) and overall survival (OS). Five-year PFS for HIF-1a positive patients was 71% versus 43% for HIF-1a negative (p=0.0187), while the 5-year OS were 75% and 54%, respectively (p=0.025). In multivariate analysis with IPI, HIF-1a remained a significant predictor for PFS (p=0.026) and OS (p=0.043). Compared with other biomarkers, HIF-1a correlated only with BCL6 (p=0.004). In terms of gene expression, we found several common gene associations of HIF-1a and the stromal-1 signature with genes predominantly involved in regulation of the extracellular matrix (e.g., BGN, COL1A2, COL5A1, and PLOD2).

Conclusion: We conclude that expression of HIF-1a protein is an important and heretofore undiscovered independent favorable prognostic factor for survival in DLBCL patients treated with R-CHOP.

235 Prospective Validation of the Uveal (Ocular) Melanoma Gene Expression Profile Prognostic Assay in Fine Needle Biopsy Specimens

J. William Harbour, Lori A. Worley, Michael D. Onken

Washington University School of Medicine, St. Louis, Missouri

Uveal (ocular) melanoma is highly lethal, with up to 50% of patients dying of metastatic disease despite successful treatment of the primary eye tumor. Identification of patients at high risk of metastasis who might benefit from adjuvant therapy has been problematic due to the lack of a highly accurately biomarker. Our research has focused on developing a highly accurate, inexpensive, and widely accessible biomarker for metastatic risk that can be performed on the primary tumor to stratify patients for inclusion in clinical trials of adjuvant therapy. We identified a gene expression profile that was highly predictive of metastatic disease. Subsequently, we developed a minimal set of 12 discriminating genes and 3 control genes that retained the full prognostic accuracy of the initial, larger gene set. The assay was then migrated from a commercial microarray to a high throughput, microfluidics, real-time PCR platform. This decreased the cost per sample about 10-fold and allowed testing of fine needle biopsy and archival specimens. The 12 gene assay was validated on several independent datasets. We then established a prospective, multi-center study to collect FNAB and archival samples and to determine the prospective performance of the prognostic assay. So far, we have recruited ten collaborating centers (now called the Collaborative Ocular Oncology Group) and have collected about 500 tumor samples, making this by far the largest study of its kind in this cancer. Among the first 146 patients who have adequate followup for analysis, 82 tumors were assigned to class 1 (low metastatic risk) and 64 tumors to class 2 (high metastatic risk). With mean followup of 17 months, 15 (10%) patients developed metastatic disease, and all had a class 2 tumor. This association between metastasis and class 2 signature was highly significant (log rank test, $P<0.0001$). Thus, the prospective data collected to date have confirmed the prognostic accuracy of the assay. We are in the process of moving the assay to an independent testing facility, which will allow the test to be performed rapidly and inexpensively and to be widely accessible to investigators around the world. We are continuing to optimize the analytical parameters of the assay through ongoing data obtained from the prospective study and are now exploring mechanisms for initiating clinical trials of adjuvant therapy using the assay to stratify high-risk patients for entry into the study.

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236 Genomic Alterations and Phenotype of Large Versus Small, High Grade Ductal Carcinoma In Situ

E. Shelley Hwang[1], Yunn-Yi Chen[2], Sandy DeVries[3], Aseem Lal[4], Joe Anderson[3], Ritu Roy[4], Frederic M. Waldman[3,4]

Departments of [1]Surgery, [2]Pathology, [3]Laboratory Medicine, [4]Cancer Center, University of California, San Francisco

Purpose: A clinically striking subtype of pure ductal carcinoma in situ (DCIS) presents as an extensive, high grade lesion that nevertheless lacks invasion. We sought to evaluate differences between those DCIS presenting as large versus small lesions while controlling for high grade, to determine if there exist phenotypic and genetic differences between the two groups that could account for the lack of invasive propensity among the large DCIS group.

Experimental Design: 52 cases of pure high grade DCIS were collected retrospectively, consisting of 27 large DCIS (>40 mm) and 25 small DCIS (<15 mm) cases. The two groups were compared on the basis of genomic copy number, assessed by array based comparative genomic hybridization (aCGH), as well as phenotype, determined by immunohistochemical analysis of ER, PR, Ki-67, p53, Cyclin D1, p16, Cox2, HER- 2, and CD68.

Results: Small DCIS presented at an older age, with greater incidence of comedo necrosis and periductal macrophage response, although these differences were not significant. Small DCIS had significantly higher ER expression, Ki-67 staining, and Cyclin D1 expression. Moreover, small DCIS had more break points and amplifications and increased copy number gains involving chromosome 8q and chromosome 20q when compared to large DCIS.

Conclusion: When controlling for grade, small and large pure DCIS show some genomic and phenotypic differences. A more thorough evaluation of these differences could help identify which DCIS has lower likelihood of progressing to invasive cancer.

237 Molecular Signatures to Improve Diagnosis and Prognostication in Peripheral T-Cell Lymphoma

Javeed Iqbal[1], Dennis D. Weisenburger[1], Timothy C. Greiner[1], Julie Vose[2], Timothy McKeithan[2], Can Kucuk[1], Huimin Geng[1], Karen Deffenbacher[1], Lynette Smith[3], Karen Dybkaer[4], Shigeo Nakamura[5], Masao Seto[5], Jan Delabie[6], Francoise Berger[7], Florence Loong[8], Wing Y. Au[8], Young-Hyeh Ko[9], Ivy Sng[10], James Armitage[2], **Wing C. Chan**[1], for the International Peripheral T-cell Lymphoma Project

Departments of [1]Pathology and Microbiology, [2]Hematology/Oncology, and [3]Preventive Medicine, University of Nebraska Medical Center, Omaha, NE; [4]Department of Hematology, Aalborg Hospital, Aarhus University Hospital, Aalborg, Denmark; [5]Aichi Cancer Center, Nagoya, Japan; [6]Norwegian Radium Hospital, Oslo, Norway; [7]Centre Hospitalier Lyon-Sud, Lyon, France; [8]Queen Mary Hospital, Hong Kong, China; [9]Samsung Medical Center, Seoul, Korea; [10]Singapore General Hospital, Singapore

Peripheral T-cell lymphoma (PTCL) is often challenging to diagnose and classify. Since most patients also have a poor outcome with standard chemotherapy, a better understanding of PTCL may lead to more effective diagnosis and therapy.

Gene expression profiling (GEP) was performed on 151 cases of PTCL and Natural Killer (NK)-cell lymphoma from the International Peripheral T-cell Lymphoma Project. In addition, NK- and T-cell lines, and normal resting and activated CD4+, CD8+ T-cells and NK-cells from healthy individuals were profiled. BRB-ArrayTools software was used to detect differentially-expressed genes, develop gene classifiers for the major PTCL entities, and identify survival predictors for AITL.

We have constructed robust molecular classifiers for angioimmunoblastic T-cell lymphoma (AITL), ALK positive anaplastic large cell lymphoma (ALK+ALCL), and adult T-cell leukemia/lymphoma (ATLL). PTCL-U was molecularly heterogeneous, but we were able to indentify a molecular subgroup with features of cytotoxic T- lymphocytes and a poor survival compared to the remaining PTCL-U cases. These classifiers reflect the biology of the tumor cells as well as their microenvironment. We also constructed a molecular prognosticator for AITL that appears to be largely related to the microenvironmental signature.

Our GEP study provided robust and biologically-meaningful classifiers for the major subtypes of PTCL and delineated a new subtype of PTCL and a molecular prognosticator for AITL. Oncogenic pathways and tumor-host interactions were also identified, and these findings may lead to better therapies and outcome in the future.

238 EBV Reactivation Syndromes in Adults Without Known Immunodeficiency

Elaine S. Jaffe[1], Stefan D. Dojcinov[2], Girish Venkataraman[1], Stefania Pittaluga[1], Mark Raffeld[1]

[1]Laboratory of Pathology, National Cancer Institute; [2]University Hospital of Wales, Cardiff, Wales

Recent studies have identified EBV+ B-cell lymphomas in the elderly related to decreased immune surveillance, with a new entity of EBV+ DLBCL of the elderly included in the WHO classification as a provisional category. We wished to identify possible precursors of EBV+ B-cell lymphoma and to characterize the spectrum of EBV-driven proliferations in an elderly population. Some 112 benign or malignant EBV+ B-LPDs were identified over a 16 year period. Patients with known lymphoma, autoimmune disease, or immunodeficiency were excluded. Cases were analyzed for histological features, immunophenotype, EBER in situ, and TCR and IgH gene rearrangement by PCR. Cases were classified as (1) hyperplasia with inc. EBV+ cells (RH); (2) nodal or (3) extranodal polymorphic EBV+ B-cell lymphoma (N-polylym/E-polylym); and (4) nodal EBV+ DLBCL. RH was diagnosed in 26 (median age 67 (45–90), and 85% of the cases were polyclonal by IgH PCR. T-cell clonality or a restricted TCR pattern was seen in 15%. Some 85% of the patients had self-limited disease, with 15% progressing to EBV+ B-cell lymphoma. There were 30 N-polylym; median age 73(48–93). Most patients had generalized LNs. IgH PCR was clonal in 36%; TCR was clonal in 23% and restricted in 15%. Clinical progression was common, seen in >85% of patients. There were 20 E-polylym; median age 77 (58–101). Most common sites were oral cavity, including gingiva, tongue, and lips; tonsillar nasopharynx, adrenal gland, and GI tract. Some 42% of cases tested were monoclonal by IgH PCR. A clonal or restricted TCR pattern was seen in 76%. Seventy % (14) of the extranodal cases were delineated as a newly identified type of lesion termed mucocutaneous ulcer. Despite atypical, often Hodgkin-like morphology, these lesions had an excellent clinical outcome with spontaneous remission in 64%, a relapsing and remitting clinical course in 21%, and complete response to treatment with no deaths due to disease in the remainder. Thirty-six were classified as EBV+ DLBCL; median age 77 (58–87). Some 75% were monoclonal by IgH PCR with restricted T-cell populations seen in 60%. All patients were treated with chemotherapy but with poor outcome and CR in only 35%.

We conclude that RH with increased EBV+ cells is frequent in the elderly but is rarely a precursor to EBV+ B-cell lymphoma. While EBV+ DLBCL has an aggressive clinical course, EBV+ mucocutaneous ulcer has an indolent natural history and may be managed conservatively. Most lesions with the exception of RH were monoclonal by IgH PCR. Clonal T-cell populations are seen in lesions of all types and may reflect a restricted antigen-driven response in an immunosenescent host.

239 8q24 Overrepresentation and Ets Gene Translocations Identify Different Prostate Cancer Subgroups

Robert B. Jenkins, Stephanie Fink, Fusheng Lan, Thomas Kollmeyer

Mayo Clinic, Rochester, MN

8q24 overrepresentation and translocations involving the ets transcription factors (ERG, ETV1 and ETV4) are frequent somatic events in prostate cancer—found in approximately 20% and 50%, respectively, of prostate cancers. While 8q24 overrepresentation is associated with a poorer prognosis, recent studies have suggested that the ets translocations are not associated with prostate cancer prognosis. Using a matched case-control design, we have recently reported ERG overexpression is not associated with systemic or biochemical progression of prostate cancer (Nakagawa et al. Plos One; 2008). We now have performed 8q24 overrepresentation analysis (by fluorescence in situ hybridization – FISH) in the same series of cases and controls. cMYC amplification and 8q24 overrepresentation was observed in 36% of cases within the systemic progression cohort, compared to 17% of the cases in the PSA progression or no evidence of disease progression groups (p-value = 0.0002; chi-square test). Furthermore, the median overall survival for men with systemic progression and 8q24 overrepresentation was 2.5 years compared to 8.5 years for those men without 8q24 overrepresentation (p-value = 0.006; log-rank test). 8q24 overrepresentation and ets gene overexpression were inversely correlated. The prevalence of 8q24 overrepresentation in tumors with any ets gene, ERG, or ETV4 overexpression was 16.0%, 17.3%, and 0.0%, respectively. Conversely, the prevalence of 8q24 overrepresentation in tumors without any ets gene, ERG, or ETV4 overexpression was 37.3%, 31.1%, and 25.1%, respectively (p-values 0.0008, 0.027, 0.003, respectively). Our results confirm prior observations showing that 8q24 overrepresentation is associated with clinically relevant prostate cancer progression events. In addition, the data demonstrate that 8q24 overrepresentation and ets gene overexpression identify at least two different subclasses of prostate cancer.

240 Investigation of an Array of Cytokines in Melanoma Patients Confirms Elevated Interferon-Gamma as a Marker of Poorer Prognosis

Jeffrey E. Lee, Daniel Y. Wang, Yuling Wang, Dawen Sui, Yun S. Chun, Dana M. McClain, Ping Liu, Roland L. Bassett, Jr., Richard E. Royal, Paul F. Mansfield, Anthony Lucci, Janice N. Cormier, Merrick I. Ross, Jeffrey E. Gershenwald

The University of Texas M. D. Anderson Cancer Center, Houston, TX

Objectives: Elevated blood levels of interferon-gamma (IFN-g) have been associated with an adverse prognosis in melanoma patients. We investigated relationships between blood levels of an array of cytokines, including IFN-g, with outcome in melanoma patients. **Methods:** Melanoma outpatients underwent blood draw for multiplex analysis for 30 cytokines (Invitrogen), including IFN-g. No patient was on systemic therapy at blood draw. Clinical data was obtained from a prospective database. Stage of disease, disease-free (DFS), and overall survival (OS) were determined from the date of blood draw. Relationships between individual plasma cytokine levels (continuous variable) and normalized summary cytokine scores, and OS were determined by Cox analysis. **Results:** The study population included 262 patients; median tumor thickness was 1.4 mm, 23% of tumors were ulcerated, and 15% of patients had an involved sentinel lymph node at presentation. At blood draw, 76% of patients were stage I–II, 21% stage III, and 3% stage IV. At a median followup of 31 months from blood collection, 25 patients had recurred and 17 had died. No elevated cytokine level predicted an improved DFS or OS. In a multivariate model that included stage, elevated blood levels of the pro-inflammatory cytokines IFN-g (P=0.01), MIP-1beta (P=0.02), and RANTES (0.01) independently predicted a shorter OS. Furthermore, elevated IFN-g was an independent predictor of melanoma recurrence (P=0.005) and death (P=0.009) in patients who presented with localized disease (stages I/II) and an independent predictor of death (P=0.005) in stage III patients. No summary cytokine score, including summary pro-inflammatory cytokine score, was superior to IFN-g at predicting DFS or OS.
Conclusions: Elevated levels of IFN-g predict a poorer outcome in melanoma patients, independent of disease stage. Investigation of mechanisms responsible for associations between IFN-g and other pro-inflammatory cytokines and outcome in melanoma patients may identify important biologic pathways responsible for disease progression and suggest novel therapies.

241 Prognostic and Predictive Gene Sets in HER2-Positive Breast Cancer: HERA Trial

Brian Leyland-Jones, Mark Bouzyk, Mark Abramovitz

Winship Cancer Institute, Emory School of Medicine

The current classification system for breast cancer (BC) is inadequate, and molecular characterization is needed in order to ensure that individualized treatment regimens are optimized for maximum benefit to the patient. Herceptin has recently been demonstrated to be effective therapy in early BC for a subset of the ~25% of women who are HER2+ and who don't develop resistance to the drug. Our long-term objectives are to (1) identify a set of genes that will be prognostic/predictive of response and relapse to Herceptin therapy in early BC; and (2) study the mechanism of resistance to Herceptin therapy in order to identify novel therapeutic targets. We will achieve the following specific aims for individualizing oncology therapeutic response via (1) determining specific signatures using microarrays and multiplexed expression arrays on an initial cohort of the HERA study (see below); and (2) subsequently validating these signatures on the remainder of the trial population. HERA, the largest of current adjuvant clinical trials, is a randomized three-arm multi-centre comparison of 1 year and 2 years of Herceptin versus no Herceptin in women with HER2+ primary breast cancer who have completed adjuvant chemotherapy. Total RNA, prepared from formalin-fixed paraffin-embedded (FFPE) tumor tissue samples, will be used for both for genome-wide microarray analysis and multiplexed gene-expression analysis. (1) Microarray analysis will use data generated from the Affymetrix human genome genechip X3P, specifically designed for use with RNA prepared from FFPE tissue. (2) The DASL assay from Illumina Inc., a robust and sensitive technique to do multiplexed expression analysis, will be performed on a custom 500-gene cancer-related panel. Analysis of data from both platforms will be integrated to come up with a prognostic/predictive set with which clinical outcome data (OS, RFS and DDFS) will be correlated. Classical prognostic and predictive approaches to the diagnosis and treatment of breast cancer are grossly inadequate; more rigorous and quantitative methods involving the latest advances in RNA-based technologies are needed. This grant application is specifically directed to achieve this aim via a 2-stage approach of both (1) determining molecular signatures of risk/benefit in HER2+ women receiving adjuvant treatment chemotherapy plus/minus Herceptin at the time of surgery and (2) validating these signatures on a large number of patients recruited in the HERA trial. If this is accomplished, it will ensure that Herceptin will be given to those women who will benefit the most, and alternative therapies will be directed to those women who would not benefit from Herceptin.

242 Global Proteomic Analysis of HNSCC Identifies Prognostic Markers

Jihyeon Lim[1,2], Nicole Kawachi[2], Peicheng Du[1], Edward Nieves[1], Abdissa Negassa[3], Thomas Belbin[2], Nicolas Schlecht[3], Margaret Brandwein-Gensler[2], Richard V. Smith[4], Ruth Hogue Angeletti[1], **Michael Prystowsky**[2]

[1]Laboratory for Macromolecular Analysis and Proteomics, [2]Department of Pathology, [3]Department of Epidemiology and Population Health, [4]Department of Otorhinolaryngology – Head and Neck Surgery, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY

Objective: The overall goal of this study is to develop independent and significant predictive measures of tumor behavior for head and neck squamous cell carcinoma (HNSCC).

Results: Site-specific analysis of global proteomic data showed an association between decrease in Annexin 1 expression and decrease in survival in oropharyngeal SCC. A validation study was performed by in situ analysis of Annexin 1 expression in all anatomic sites in 96 patients with HNSCC. While the various Annexin 1 expression assessments (i.e., nuclear, membranous, cytoplasmic) for the most part showed that those with the high level of expression have a lower hazard rate of death or progression or local regional recurrence (LRR), only the association with cytoplasmic Annexin 1 on LRR reached statistical significance. While the result is interesting, the small number of events (13) on which the analysis is based calls for cautious interpretation (i.e., this result needs to be confirmed in a larger dataset and should not be considered definitive). Additional 2D LC-MS analysis of HNSCC samples (47 oropharyngeal, 50 oral cavity, and 25 larynx) were performed. The 2D LC-MS data from 43 cases of oropharyngeal SCC were analyzed to produce a group of peptides that correlate with clinical outcome. Two lists of peptides, when considered together in a classification algorithm, were produced that result in an error rate of about 12%. One list is associated with disease progression and the other with survival. The number of peptides (25) is based on practical consideration such as ease of implementation in a clinical setting. For instance, the breast cancer assay, Oncotype Dx, has 21 genes.

Conclusion: The results strongly suggest that a small number of peptides can be used to develop a predictive model that can discriminate disease severity at initial diagnosis as well as clinical outcome prospectively. RNA expression profiling of primary HNSCC specimens have also been performed. We will integrate the proteomic data with the RNA expression data to identify the best potential prognostic biomarkers to develop clinical diagnostics. In the future, we will again refine and validate the predictive importance of peptide discriminators by analyzing additional HNSCC samples, part of which serves as a “test set“ with respect to tumor behavior and clinical outcome as well as obtain more precise estimates of effect.

243 Translational Research to Practice for Gastric Cancer in China: Molecular Classification and Outcome Prediction Based on Genome and Proteom Profiling

Youyong Lu

Laboratory of Molecular Oncology, Peking University School of Oncology, Beijing Cancer Hospital/Institute

We have generated gene and protein expression profilings to approach cellular and molecular mechanism and to discover diagnostic and predictive biomarkers of gastric and colorectal cancer. Based on these gene expression profiles, we explored the characteristics of molecular changes and their potential for clinical significance.

As our present data and systemic analyses for gene expression pattern, pathway distribution, gene function category, biosignature, and clinical significance shows, we have been able to document the entire gene expression profiles for intestinal- and diffuse-type GC and normal appearing tissues (NATs) matched tumors. A group of specific or typical genes were identified as having dramatic changes in tumors and NATs compared with the verified normal samples. Among these genes, at least three gene sets were analyzed using pathway analysis tools and integrated with biological assay data to construct a network for GC carcinogenesis. The results show that these genes are involved in several well-studied signaling pathways associated in development and progression of gastric cancer. A typical result indicated that over-expression of MMP11 at mRNA and protein level was consistently detected in cell lines and primary tumors compared with matched normal tissues. Importantly, serum MMP11 levels were also significantly elevated in GC patients compared with those of the control subjects, and the positive expression was well correlated with metastasis and recurrence in GC patients.

As its pilot study, we have generated primary data of its genomic alterations, in combination and comparison with the gene expression profiles. Dramatic correlations have been observed between the gene expression profiles and DNA Copy Number Variations (CNVs), with statistically significant differences between tumors of Stages I–II and III–IV GC. We have also defined a group of specific gene or miRNA alterations, which could be associated with metastasis and recurrence in GC patients. Taken together, we have been the first to provide a systematic analysis for comprehensive gene expression profile integrated with microRNA, genomic, or proteomic analysis for gastric cancer. Additionally we have defined a group of genes associated with development and prognosis in GC.

244 Biomarkers for Risk and Target Identifications in Pediatric AML

Soheil Meshinchi[1,2], Todd A. Alonzo[2,3], Alan S. Gamis[2,4], Franklin O. Smith[2,5], Michael R. Loken[2,6], Gregory H. Reaman[2,7]

[1]Fred Hutchinson Cancer Research Center, Seattle, WA; [2]Children's Oncology Group, Arcadia, CA; [3]University of Southern California, Los Angeles, CA; [4]Children's Mercy Hospital & Clinics, Kansas City, MO; [5]Cincinnati Children's Hospital Medical Center, Cincinnati, OH; [6]Hematologics Inc. Seattle, WA; [7]The George Washington University School of Medicine and Health Sciences

The Children's Oncology Group (COG) is an international clinical trials cooperative group supported by the National Cancer Institute with the unique ability to enroll nearly all pediatric patients in North America, resulting in standard of care through state of the art treatments for children with cancer. As part of efforts to optimize outcome in children with AML, COG Myeloid Disease Committee has adopted a risk-based therapy in AML in order to decrease relapse in high-risk patients and minimize toxicity in those expected to have a more favorable outcome. Identification of biomarkers for risk and therapeutic target identification has been incorporated into COG AML phase III trials. As part of these efforts, FLT3/ITD with high allelic ratio (high ITD-AR) has been identified as a marker of high-risk disease in AML. As a result, high ITD-AR has been incorporated as a clinical risk factor in the current COG phase III AML trial, where those with high ITD-AR will be allocated to receive allogeneic stem cell transplant in first CR, with plans to incorporate FLT3 inhibitors into their therapy following current feasibility testing. Further molecular profiling of genes involved in myeloid pathogenesis have CEBPA and NPM mutations to be significantly associated with favorable outcome with numerous other genes under investigation for their association with disease outcome. In addition to molecular risk factors, we have demonstrated MRD is highly associated with disease outcome in patients without known prognostic markers, where patients in morphologic remission who have evidence of disease by MDF are at high risk of relapse. We have also demonstrated that high-level expression of adhesion molecule VLA-4 is associated with disease outcome in patients with standard risk AML. As an integrated aim in the current trial, we will optimize the utility of MDF (threshold, time point, etc.) to identify patients in morphologic remission with minimal residual disease (MRD) who are at high risk of relapse. Additional efforts are underway for evaluation of molecular MRD in those with specific cytogenetic markers, including t(8;21), inv(16) and t(9;11). MRD will be merged with molecular MRD, cytogenetic profiles and mutational profile and will be collectively assessed in order to define the most accurate risk profile for the largest number of patients. The data generated will be used for risk identification as part of risk-based therapy in the next phase III trial.

245 Immunostaining for CD10, BCL6, MUM1, LMO2, and BCL2 to Identify Favorable Survival for Molecular Subtypes of Diffuse Large B-Cell Lymphoma in a Population-Based Study During the Pre-Rituximab Era

Lindsay M. Morton[1], James R. Cerhan[2], Patricia Hartge[1], Mohammad A. Vasef[3], Vishala Neppali[4], Yasodha Natkunam[5], Ahmet Dogan[2], Ronald Levy[5], Izidore S Lossos[5], Wendy Cozen[6], Scott Davis[7], Mary Jean Schenk[8], Matthew J. Maurer[2], Charles F. Lynch[4], Nathaniel Rothman[1], Nilanjan Chatterjee[1], Kai Yu[1], Louis M. Staudt[9], Dennis D. Weisenburger[10], Sophia S. Wang[1]

[1]Division of Cancer Epidemiology and Genetics, National Cancer Institute (NCI), National Institutes of Health (NIH); [2]Mayo Clinic College of Medicine; [3]University of Arizona; [4]University of Iowa; [5]Stanford University School of Medicine; [6]Keck School of Medicine, University of Southern California; [7]Fred Hutchinson Cancer Research Center and University of Washington; [8]Barbara Ann Karmanos Cancer Institute, Wayne State University; [9]Center for Cancer Research, NCI, NIH; [10]University of Nebraska Medical Center

Diffuse large B-cell lymphomas (DLBCL) are heterogeneous. Studies of gene expression profiles have distinguished DLBCL malignancies by cell of origin [germinal center (GCB-DLBCL) vs. non-germinal center B-cell (non-GCB-DLBCL)], with distinct pathogenetic mechanisms and better prognosis for patients with GCB-DLBCL. Clinical application of this work requires the use of routine, inexpensive techniques such as immunohistochemistry, but attempts to classify DLBCL subtypes using immuno-histochemistry have yielded inconsistent results. We evaluated the prognostic significance of five immunohistochemically determined markers expressed in germinal center (CD10, BCL6, LMO2) and activated B-cells (MUM1, BCL2) in 214 patients with DLBCL. Patients aged 20–74 years without known HIV infection were identified from four population-based SEER registries during 1998–2000 and followed for overall survival until 2008. Cox regression models were used to estimate hazard ratios, accounting for age, demographic, and clinical factors. The study population was predominantly white (90%) and male (59%), and the median age at DLBCL diagnosis was 59 years. Clinically, 27% of patients had B-symptoms, and 88% received initial chemotherapy (pre-rituximab era). During followup, 69 (32%) patients died. Of the 5 markers evaluated individually, BCL2 expression (>90%) was most strongly associated with poorer prognosis: the 41 (19.2%) patients with BCL2-positive DLBCL had significantly poorer overall survival than patients with BCL2-negative DLBCL (41% vs. 74%; p=0.001). We classified 112 (52%) cases as GCB-DLBCL using the algorithm of Hans et al. (Blood 2004; CD10≥30% or CD10 <30% +BCL6 ≥30% +MUM1<30%), but these patients did not have significantly better overall survival than patients with non-GCB-DLBCL (71% vs. 64%; p=0.699). Using the same algorithm but deriving the optimal cutoff for immunostain positivity from our survival data, we classified 112 (52%) cases as GCB-DLBCL (CD10>70% or CD10≤70%+BCL6>10%+MUM1≤60%); these GCB-DLBCL patients had non-significantly better overall survival (73% vs. 62%; p=0.111). No algorithm with all five markers significantly discriminated prognostic groups in our study. Although identifying DLBCL molecular subtypes holds great promise for identifying patients with favorable prognosis and could have therapeutic implications, we conclude that additional research is needed to establish reliable fixed tissue markers for clinical risk stratification.

246 Multimarker Analysis of Breast Cancer Patients Using Weighted Correlation Network

Angela P. Presson[1,2], Nam K. Yoon[3], Vei Mah[3], Mohammad Alavi[3], Erin Maresh[3], Lee Goodglick[3,5], **David Chia**[3,5], Steve Horvath[1,4,5]

Departments of [1]Biostatistics, [2]Pediatrics, [3]Pathology and Laboratory Medicine, and [4]Human Genetics, and the [5]Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, UCLA, Los Angeles, CA

Breast cancer is the most common type of cancer in women. While survival rates are improving, nearly one in four women is expected to acquire the disease within her lifetime. Our goal is to identify one or more proteins that yield meaningful information on the development and/or progression of this disease. To do this, we have constructed a high-density breast cancer tissue microarray (TMA) consisting of 1,816 spots from 212 individuals and have analyzed the expression level and localization of 26 putative tumor biomarkers on this platform. We used a Weighted Co-expression Network Analysis (WCNA) to cluster the patients according to their marker expression levels. Spearman correlations were used to relate the marker-defined patient groups with survival and clinical traits. Kaplan-Meier plots were used to identify clinical traits that were significant survival predictors, and these traits were included in a multivariate analysis. A Cox proportional hazards model was used to assess the predictive value of the patient groups in comparison to the clinical traits. Classification and regression tree (CART) methods were used to find a subset of markers that could approximately define these patient groups. Our network analysis of these 26 cancer markers identified three patient groups. Group 1 had the highest death rate (50%), while the death rate in Group 2 was 28.6% and in Group 3 was 5.4%. These multimarkers define unique subgroups that were not necessarily correlated with known clinical traits. In addition, none of the clinical traits were significantly different between the groups (p-values > 0.1). Patient group was the only significant survival predictor in the multivariate analysis (p-value = 0.037). CART identified three proteins of the original 26 total, which were most relevant in defining Groups 1, 2 and 3. These were P53, Na-K ATPase ß chain, and TGF-ß receptor II. In conclusion, this study has defined a novel approach for analyzing multiple biomarkers on a population basis, with a goal of defining meaningful and clinically significant subpopulation of cancer with distinct outcomes profiles.

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247 Gene Expression Profiling to Predict Cancer Development in Oral Preneoplastic Lesions

Pierre Saintigny[1], Li Zhang[2], You-Hong Fan[1], Adel K. El-Naggar[3], Vali Papadimitrakopoulou[1], Lei Feng[2], J. Jack Lee[2], Edward S. Kim[1], Waun Ki Hong[1], Scott M. Lippman[1], Li Mao[1,4]

[1]Department of Thoracic/Head and Neck Medical Oncology, [2]Department of Biostatistics and Applied Mathematics, [3]Department of Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, TX; [4]University of Maryland Dental School, Baltimore, MD

Purpose: The risk of malignant transformation of oral preneoplastic lesion (OPL) is difficult to assess. We have reported that podoplanine and deltaNp63 expression are associated with oral cancer development. The purpose of this study was to demonstrate the value of gene expression profiling to predict oral cancer development in patients with OPL. **Experimental Design:** Gene expression was studied using Affymetrix Gene 1.ST platform in 91 of 162 patients included in a phase III clinical trial comparing retinyl palmitate alone or plus beta-carotene with low dose 13-cis retinoic acid. Among these 91 patients, 35 developed oral cancer and 56 did not develop oral cancer during a median followup of 7 years. A boosting approach (R package CoxBoost) was used to identify expression profiles of genes that are associated with oral cancer-free survival and to develop a prognostic model in combination with clinical covariates like histology at baseline, podoplanin and deltaNp63 expression. **Results:** We found 681 probesets significantly associated with risk of developing oral cancer (single-variate Cox-ph model, p-value<0.001). The most significant genes associated with a high risk include DNMT3B, cell adhesion modulators (SPP1, IBSP, DSPP, DMP1), cell cycle modulators (CCAR1, CCNL1), topoisomerase I, serine/threonine kinase 3, and TP63. Genes associated with a low risk to develop oral cancer include CDKN1B, TIMP2, and XRCC1. We obtained a prognostic model using CoxBoost method applied to the microarray gene expression and setting the clinical covariates (i.e., histology at baseline, podoplanin and deltaNp63 expression) as mandatory predictors. The prognostic model includes 42 probesets that have a significant overlap with the genes identified from single-variate Cox-ph models, including DNMT3B. To evaluate the performance of the model, we used the .632+ bootstrap method and prediction error curve estimates. Compared to a model that used only the clinical covariates, our prognostic model showed a marked improvement in predicting risk of oral cancer. **Conclusion:** Gene expression profiles may improve the prediction of oral cancer development in patients with OPL beyond clinical covariates. Validation in an independent dataset is warranted. Epigenetic tumorigenesis mediated by DNMT3B could be an early event in OPL, potentially targetable in the chemoprevention setting.

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248 Association of VEGF and VEGFR-2 Genetic Polymorphisms With Outcome in E2100

Bryan P. Schneider[1], Molin Wang[2], Milan Radovich[1], George W. Sledge[1], Sunil Badve[1], Ann Thor[3], David A. Flockhart[1], Bradley Hancock[1], Nancy Davidson[4], Julie Gralow[5], Maura Dickler[6], Edith A. Perez[7], Melody Cobleigh[8], Tamara Shenkier[9], Susan Edgerton[3], Kathy D. Miller[1]

[1]Indiana University School of Medicine, Indianapolis, IN; [2]Dana Farber Cancer Institute, Boston, MA; [3]University of Colorado Health Sciences Center, Denver, CO; [4]Johns Hopkins , Baltimore, MD; [5]University of Washington & Fred Hutchinson Cancer Research Center, Seattle, WA; [6]Memorial Sloan-Kettering Cancer Center, NY; [7]Mayo Clinic, Jacksonville, FL; [8]Rush University Medical Center, Chicago, IL; [9]British Columbia Cancer Agency, Vancouver, BC, Canada

Background: No biomarkers have been identified to predict outcome with the use of an anti-angiogenesis agent for cancer. Vascular endothelial growth factor (VEGF) genetic variability has been associated with altered risk of breast cancer and variable promoter activity. Therefore, we evaluated the association of VEGF genotype with efficacy and toxicity in E2100, a phase III study comparing paclitaxel versus paclitaxel with bevacizumab as initial chemotherapy for metastatic breast cancer.

Methods: DNA was extracted from tumor blocks of patients from E2100. Some 363 cases were available to evaluate associations between genotype and outcome. Genotyping was performed for selected polymorphisms in VEGF and VEGF-receptor 2. Testing for associations between each polymorphism with efficacy and toxicity was performed.

Results: The VEGF -2578 AA genotype was associated with a superior median overall survival (OS) in the combination arm when compared to the alternate genotypes combined; hazard ratio 0.58 (95% C.I.=0.36, 0.93; p=0.023). The VEGF -1154 A allele also demonstrated a superior median OS with an additive effect of each active allele in the combination arm but not the control arm; hazard ratio 0.62 (95% CI= 0.46, 0.83; 0.001). Two additional genotypes, VEGF -634 CC and VEGF -1498 TT, were associated with significantly less grade 3/4 hypertension in the combination arm when compared to the alternate genotypes combined (p=0.005 and p=0.022, respectively).

Conclusion: Our data support an association between VEGF genotype with median OS as well as with grade 3/4 hypertension when using bevacizumab in metastatic breast cancer.

249 An Experimentally Derived Metastasis Gene Expression Profile Predicts Recurrence and Death in Colon Cancer Patients

J. Joshua Smith[1], Natasha G. Deane[1], Fei Wu[1], Nipun B. Merchant[1], Bing Zhang[7], Aixiang Jiang[1], Pengcheng Lu[1], J. Chad Johnson[1], Carl Schmidt[1], Christina M. Edwards[1], Steven Eschrich[3], Christian Kis[1], Shawn Levy[1], M. Kay Washington[1], Martin J. Heslin[2], Robert J. Coffey[1], Timothy J. Yeatman[3], Yu Shyr[1], **R. Daniel Beauchamp**[1]

[1]Vanderbilt University Medical Center, Nashville, TN; [2]University of Alabama School of Medicine-Birmingham, Birmingham, AL; [3]H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

Background and Aims: Staging inadequately predicts metastatic risk in colon cancer patients. We asked whether a gene expression profile derived from invasive murine colon cancer cells that were highly metastatic in an immunocompetent mouse model could be used to identify colon cancer patients at risk for cancer recurrence and death.

Methods: Primary tumor gene expression profiles from 55 colorectal cancer patients from Vanderbilt Medical Center (VMC) were used as the training dataset, and expression profiles of 177 patients from the Moffitt Cancer Center were used as the independent dataset. A 300 gene metastasis-associated expression profile developed from the mouse model was refined using the VMC colon cancer gene expression profiles to identify a 34-gene classifier associated with high risk of metastasis and death from colon cancer. A recurrence score derived from the biologically based classifier was tested in the Moffitt colon cancer dataset.

Results: A high recurrence score was significantly associated with increased risk of metastasis and death from colon cancer across all pathological stages and specifically in stage II and stage III patients. The recurrence score was shown to independently predict risk of cancer recurrence and death in both univariate and multivariate models. For example, the subgroup of high score stage II patients had a high risk of cancer recurrence (hazard ration=13.1; p=0.01; 95% CI=1.7 to 103.1) and in stage III patients with a high score also were at significantly increased risk of recurrence (hazard ratio=4.7; 95% CI=1.566–14.05). Furthermore, the recurrence score identified a subgroup of stage III patients whose 5-year recurrence-free survival was >88% and for whom adjuvant chemotherapy did not provide improved survival.

Conclusion: Our biologically based gene expression profile yielded a potentially useful classifier to predict cancer recurrence and death independently of conventional measures in colon cancer patients.

250 Molecular Differences in Primary Pancreatic Tumors

Jeran Stratford[1], Judy M. Andersen[2], J. S. Marron[1], Keith A. Volmar[1], Elizabeth D. Routh[1], Laura Caskey[1], Channing J. Der[1], Benjamin F. Calvo[1], Mark Talamonti[3], Christine Iacobuzio-Donahue[4], David J. Bentrem[3], Charles M. Perou[1], Michael A. Hollingsworth[2], **Jen Jen Yeh**[1]

[1]Lineberger Comprehensive Cancer Center, University of North Carolina Chapel Hill; [2]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center; [3]Robert H. Lurie Comprehensive Cancer Center, Northwestern Memorial Hospital; [4]Johns Hopkins Medical Institutions

Pancreatic ductal adenocarcinoma (PDAC) remains a lethal disease with a worldwide incidence of 232,000 in 2008 and a nearly equal number of deaths. For the 20% of patients with localized disease, median survival after surgery is 17 months, with many patients recurring shortly after surgery. However, 12% of resected patients can survive more than 5 years, suggesting that there is a need to better stratify patients who may benefit from surgical therapy and study the molecular changes associated with the different biology of these tumors.

We compared gene expression profiles of primary tumors of patients who died of metastatic PDAC and of patients with resectable disease. We evaluated these differences in three independent groups of 19 patients from Stanford University, 34 patients from the Johns Hopkins Medical Institutions, and 49 patients from Northwestern University.

Using significance analysis of microarrays and a 10-fold cross-validation approach, we identified a six gene signature from our training set with a predicted accuracy of 96%. Applied to the three test sets from independent institutions, the six gene signature predicted survival of patients with resectable PDAC better than current clinical prognostic factors across institutions.

Our six gene signature is independently predictive of outcome in patients with resectable PDAC and suggests that the differences in biology of this disease may be inherent in the primary tumors. Studying these genes may provide insight into the aggressive biology of this disease. As more therapies become available for PDAC, our gene signature may be used to better tailor therapy for patients.

251 Tissue Biomarkers Predicting Endometrial Cancer Stage and Recurrence: A Paradigm for Identifying Patients Who Would Benefit Most From Surgical Staging and Adjuvant Treatment

Shannon Westin[1], Diana Urbauer[1], Peter Mueller[1], David Loose[2], **Russell Broaddus**[1]

[1]The University of Texas M. D. Anderson Cancer Center, Houston, TX; [2]University of Texas-Houston Medical School

Background: Ongoing controversy exists regarding the surgical management of endometrial cancer patients. Identification of women who would benefit most from complete surgical staging is unresolved. Many patients with endometrial cancer are poor candidates for extended surgeries due to obesity, hypertension, and diabetes. Intra-operative frozen section is not universally available and is not always reliable. The goal of our study is to determine if a panel of molecular markers can potentially assist in the decision to perform complete surgical staging on women diagnosed with endometrial cancer. Given the relationship between estrogen exposure and endometrial cancer, especially low-grade endometrial cancer, we hypothesized that such a biomarker panel will provide a clinically useful biomarker score to assist in the decision to perform complete surgical staging on women diagnosed with endometrial cancer. **Design:** Microarray was performed in baseline and post-treatment endometrial biopsies from women taking estrogen-based HRT to identify genes regulated by estrogen. The expression six genes most strongly induced by estrogen (RALDH2, sFRP1, sFRP4, EIG121, IGF-I, and IGF-IR) were quantified by qRT-PCR in 56 endometrioid-type endometrial carcinomas. Expression data was compared to clinico-pathologic characteristics, and an unsupervised cluster analysis was performed. Time to recurrence by cluster was analyzed using the Kaplan-Meier method. A receiver operating characteristic (ROC) curve was generated to determine the clinical utility of the panel to predict endometrial cancer stage. **Results:** Unsupervised cluster analysis revealed two distinct groups based on gene expression. The low expression cluster had a recurrence rate 4.35 times higher than that of the high expression cluster. Included in the low cluster were two patients with grade 1 endometrioid tumors who later had recurrence. ROC analysis allowed for the prediction of endometrial cancer stage Ic or higher with a false negative rate of only 4.5% based on level of gene expression. **Conclusion:** This biomarker panel was highly accurate in stratifying endometrial cancer patients into low risk (stage Ia or Ib) versus high risk (stage Ic or higher) groups and could also identify those at-risk for recurrence. The biomarker panel may therefore help to better identify the patients who would most benefit from extensive endometrial cancer staging.

252 Prognostic Markers for Ovarian Cancer

Kwong-Kwok Wong[1], Michael J. Birrer[2], Samuel C. Mok[1]

[1]The University of Texas M. D. Anderson Cancer Center; [2]Massachusetts General Hospital

Ovarian cancer is the fifth most common form of cancer in women in the United States, accounting for 4% of the total number of cancer cases and 25% of those cases occur in the female genital tract. Because of its low cure rate, it is responsible for 5% of all cancer deaths in women. It was estimated that 14,600 deaths were caused by ovarian cancer in 2009. A majority of ovarian cancer cases are detected at an advanced stage (where metastases are present beyond the ovaries) and are rarely curable. Though most patients die within 2 years of diagnosis, a subset of patients develop a more chronic form of ovarian cancer and may survive 5 years or more with treatment. Using a newly developed expression tag oligonucleotide array CGH and microdissected tumor tissue samples, we have recently identified 12 CGH segments that are associated with overall survival in patients with high-grade advanced stage serous adenocarcinoma. We showed that DNA copy numbers of multiple genes in the 12 CGH segments were significantly correlated with transcription levels of the genes, which were identified by transcriptional profiling of RNA isolated from the same set of tumor tissue samples. Using an independent set of high-grade late stage serous cancer specimens, validation studies on one of the genes FGF1 located on 5q31 showed that mRNA copy number was significantly correlated with DNA copy number and protein expression levels, and both FGF-1 mRNA and protein levels were significantly associated with overall patient survival. These data suggest that the combined array CGH and expression profiling strategy can successfully identify genetic based biomarkers with prognostic value. Our study focuses on developing a genetic based prognostic model for high-grade advanced stage serous adenocarcinoma. Genes located in 12 CGH segments that are significantly associated with overall survival in patients with high-grade advanced stage serous ovarian cancer are currently being validated. These markers could be developed to detect aggressive cancers and should allow the identification of a “druggable” target within the amplicon, which can stratify patients into prognostic groups and allow for this finding to be incorporated into the design of phase III clinical trials.

253 Tobacco Use Is Associated With Risk of Distant Metastases, Tumor Recurrence, and Death in Patients With Human Papillomavirus (HPV)-Positive (+) Squamous Cell Carcinoma of the Oropharynx

Francis Worden, Jessica Hooton, Bhavna Kumar, Felix Feng, Julia Lee, Avraham Eisbruch, Gregory Wolf, Mark Prince, Jeffrey Moyer, Theodoros Teknos, Douglas Chepeha, Jay Stoerker, Heather Walline, Jonathon McHugh, Katrina Cordell, Preston Ward, Susan Urba, Carol Bradford, Thomas Carey

Comprehensive Cancer Center, University of Michigan, Ann Arbor, MI

Background: Chemoradiotherapy for HPV (+) squamous cell carcinomas of the oropharynx cancer is associated with a more favorable prognosis than HPV-negative (–) oropharynx cancer. However, the interaction of HPV and tobacco in terms of etiology and disease progression remains unclear. HPV (+) oropharynx cancer patients were prospectively studied to determine if tobacco use was a key variable in discriminating which patients would develop distant metastases, local regional recurrences, or second primary tumors. **Methods:** From 1999–2007, 124 patients with stage III/IV oropharynx cancer were enrolled in one of two chemoradiotherapy trials. Tumor specimens were analyzed for HPV presence and type. Use of tobacco, determined via self-reporting and chart review, was recorded as both continuous (number of pack-years) and categorical (never, former, and current) variables. Former tobacco users were described as those who quit ≥20 years prior to diagnosis, and current smokers were those who were presently using tobacco, including those who quit < 20 years prior to diagnosis. Tobacco use and HPV status were analyzed with respect to survival and the development of distant metastases, local recurrences, or second primary tumors. **Results:** Of the 124 patients, 100 (81%) were HPV (+), 22 of which developed disease progression (22%). Twenty-four were HPV (–), 12 of which had disease progression (50%). Seventeen of 124 patients (14%) developed distant metastases [12 HPV (+), 5 HPV (–)]. Nine of 124 (7%) developed local recurrences [5 HPV (+), 4 HPV (–)], and 8 of 124 (7%) developed second primary tumors [5 HPV (+), 3 HPV (–)]. Thirty-two HPV (+) patients were never-tobacco users, 88% (28/32) of whom remain alive with no evidence of disease; 3 died from other causes, and 1 died of lung metastases from oropharynx cancer. Sixty-eight were HPV (+) and had tobacco exposure. Of 46 former tobacco users, 37/46 (80%) are living. Of 22 HPV (+) current tobacco users, 68% (15/22) are alive and 36% (8/22) have developed disease progression. Seventeen of the 24 HPV (–) patients were current tobacco users, 47% (8/17) of which developed disease progression. The risk of recurrence was greater amongst the current tobacco users when compared to never-tobacco users (HR 5.2 [1.1–24.4]), p=0.038. HPV (+) current users of tobacco were more likely to die from oropharynx cancer compared to HPV (+) never users of tobacco (HR 7.2 [0.88-58.4]), p=0.07). **Conclusions:** Never-tobacco users with HPV-positive oropharynx cancer have improved survival and reduced risk of disease recurrence compared to HPV-positive oropharynx patients who currently use tobacco.

254 The Prognostic Significance of Lymphatic Invasion in Primary Melanoma

Xiaowei Xu, Lianjun Chen, Wei-Ting Hwang, Peter Dawson, DuPont Guerry, Pat VanBelle, David Elder, Lynn Schuchter, Phyllis Gimotty

University of Pennsylvania

Background: Lymphatic invasion (LI) is an under-observed phenomenon in primary malignancies that can be better detected by immunostaining and that may associate with prognosis. In this study we sought to test the hypothesis that LI was associated with melanoma-specific survival (MSS) and was an independent prognostic factor. **Methods:** This study included 277 patients with stage I/II melanomas in vertical growth phase (VGP) who had at least 10 years of followup. The log-rank test was used to test the study hypothesis — 72 melanoma-specific deaths were needed for 80% power to detect an odds ratio of 2.1. Paraffin sections were stained with antibodies to podoplanin (lymphatic vessels) and S-100 (melanoma cells) to identify LI. Univariate and multivariate Cox models were used to evaluate the prognostic significance of LI. An independent cohort of 106 similar patients was used for validation of the 10-year MSS rates. **Results:** LI was observed in 44.5% (95% CI: 38.6% – 50.4%) of the melanomas, and its presence was significantly associated with thickness, mitotic rate, gender, age, and ulceration. The Kaplan-Meier survival curves for those with and without LI were significantly different (log-rank test p=0.022). The final multivariate model for time to MSD identified four independent prognostic factors: thickness (HR=1.5, p<0.001), ulceration (HR=2.2 p=0.013), site (HR=3.9, p<0.001), and LI (HR=1.9, p=0.015). These factors were used to define a prognostic tree with five risk groups defined by melanomas that were thin (≤1.0mm) with no LI or ulceration; thin with LI but no ulceration; 1–3mm with no ulceration; 1–3mm with ulceration; and >3mm. Respectively, MSS rates were 100%, 88.6%, 77%, 48%, and 42%. In the validation set, observed 10-year MSS rates in each risk group were not significantly different from those predicted from the survival curves for the tree-based risk groups. **Conclusions**: LI is an independent prognostic factor for MSS. Among patients with thin melanomas without U, the 10-year MSS was lower for those patients with LI (89%, 95% CI=78% – 99%; n=41) compared to those without (100%, n=78). LI is an important prognostic factor that needs further validation in a population of patients from the sentinel node biopsy era.

255 Circulating Tumor Cell Capture and Analysis in a Multi-Center Prostate Cancer Trial

Tong Xu[1], Bo Lu[2], Roy Lau[1], Justin Allen[1], Louis M. Fink[3], Oscar Goodman[3], Beth Grobaski[3], Anthony Williams[4], Ram Datar[4], Bryan Goldman[5], Cathy M. Tangen[5], Nicholas J. Vogelzang[3], Yu-Chong Tai[2], Richard J. Cote[4], David I. Quinn[1], **Amir Goldkorn**[1]

[1]University of Southern California Keck School of Medicine & Norris Comprehensive Cancer Center, Los Angeles, CA; [2]California Institute of Technology, Pasadena, CA; [3]Nevada Cancer Institute, Las Vegas, NV; [4]University of Miami Miller School of Medicine, Miami, FL; [5]Southwest Oncology Biostatistical Center, Seattle, WA

Prostate cancer biomarkers are urgently needed to better inform treatment decisions. Recent studies have demonstrated that quantification of peripheral blood circulating tumor cells (CTCs) predicts response to therapy and overall survival in advanced prostate cancer. However, present methods for CTC collection are limited by low yield, complex techniques, and expensive equipment, and they provide little phenotypic information about the CTCs themselves. To address these limitations, we have developed a new microfilter device that is fitted to an ordinary syringe and reliably traps and enriches the CTC population from peripheral blood, enabling enumeration and further study of these cells. We hypothesize that our microfilter can serve as a simple yet reliable new predictive platform for CTC collection, quantification, and phenotypic analysis in a large clinical trial setting. To test this hypothesis, we have activated a correlative study that piggybacks onto S0421, a SWOG cooperative group protocol studying atrasentan in combination with docetaxel in castration resistant prostate cancer. At three time points before and during treatment pre-designated by S0421, blood samples are drawn and processed through the microfilter device, and the captured CTCs are analyzed to address the following specific aims: (1) Do absolute CTC counts and post-treatment changes in CTC counts accurately predict clinical outcome and response to therapy? As further validation of microfilter CTC capture, parallel samples are analyzed using the FDA approved Cell Search CTC collection platform; (2) Does the expression of relevant biomarkers on microfilter-trapped CTCs predict clinical outcome and response to therapy? Specifically, we are assessing endothelin receptor A for atrasentan response, type III beta-tubulin for docetaxel response and CD44 for an aggressive progenitor/metastatic phenotype; and (3) Does the presence and level of telomerase activity (an established cancer marker) in microfilter-enriched cells correlate with the presence and number of captured CTCs, and can it be used to predict clinical outcome and response to therapy? In summary, our goal is to determine if the quantity and characteristics of CTCs captured on our novel platform can predict clinical outcome and response to therapy, thus enhancing our ability to assess therapeutic efficacy in real time and contributing to optimized, evidence-based, individualized patient management.

256 Rapid Autopsy Program and Tissue Resource for Pancreatic Cancer

Judy M. Anderson, Michael A. Hollingsworth

Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha

Pancreatic cancer is lethal and the fourth leading cause of cancer deaths in North America. Investigators working to understand the biology of the disease are impeded by a paucity of human tissue specimens, particularly from metastatic deposits. The majority of patients with pancreatic adenocarcinoma have metastatic disease at the time of diagnosis and rarely undergo other invasive procedures that would permit harvesting fresh tumor specimens. Diagnosis by percutaneous fine needle aspiration biopsy yields limited quantities of tissue that are typically insufficient for research purposes, and surgical resection provides limited samples for research and lacks metastatic and normal tissue for comparison. To address this need, we developed an organ harvest/rapid autopsy program in which patients who die with pancreatic cancer donate their organs for research purposes. These autopsies are performed within 1–3 hours of death to prevent tissue degradation. Briefly, tissues are procured by the pathologist, pathology assistant, and a 12-member volunteer team of students, postdocs, and technologists who are on call at UNMC. The pathologist locates and procures samples of primary tumor and metastatic lesions; designated organ sections are assigned to specific teams, who process the specimens by dissection. Specimens are snap frozen in liquid nitrogen and placed on dry ice until their final storage in –80°C freezers. In addition to obtaining neoplastic tissue and adjacent benign pancreas, we also collect tissue from all possible involved and uninvolved organs for comparative analyses. Once the procedure is completed, the Pancreas SPORE Tissue Bank technologist enters all specimen information and freezer locations into the shared Freezerworks database. Rapid autopsy samples have been evaluated by a number of procedures, including Northern blot, Southern blot, PCR, SDS-PAGE, immunohistochemistry, in situ hybridization, immunofluorescence, and other analyses with known probes. All samples to date have been of outstanding quality using these types of analyses. We currently have over 30,000 samples (frozen), representing 30 kilograms of harvested tissue, from conducting 33 rapid autopsies. The availability of tissue from primary and metastatic pancreatic malignancies is a unique and valuable resource that is available to any investigator with meritorious and feasible projects.

257 Integrated Biochip Sensors for Detection of Cancer

Rashid Bashir[1], Ashraf Alam[2], Donald E. Bergstrom[3], Susan E Clare[4], Luke P. Lee[5]

[1]Electrical and Computer Engineering, Bioengineering, Micro & Nanotechnology Laboratory, University of Illinois at Urbana-Champaign; [2]Electrical and Computer Engineering and [3]Medicinal Chemistry and Molecular Pharmacology, Birck Nanotechnology Center, Purdue University, West Lafayette, IN; [4]Department of Surgery, Indiana University School of Medicine, Indianapolis, IN; [5]Bioengineering, University of California, Berkeley

The recent technological advances in top-down silicon nanotechnology and micromachining of semiconductor materials present themselves with new opportunities for small, sensitive, one time use, point of care diagnostic devices capable of rapid and highly accurate analysis of samples of body fluid. Nanosensors and microfluidic biochips have been successfully demonstrated for the detection of proteins in fluids, but have yet to be realized in a robust array format, with detection of multiple proteins, which can be used in preclinical studies or clinical samples. In this proposal, we have assembled a truly comprehensive team of interdisciplinary researchers with the goal of applied and translational multidisciplinary research for designing and producing robust top-down silicon-based field-effect nano-sensor platform technologies integrated in one-time-use point-of-care diagnostic biochips, functionalized with multiple antibodies, for the ex-vivo label detection of cancer proteins from cell lysate from breast aspirates. The successful end goal of the project would be to assess the use of these sensors for cancer diagnostics and theragnostics (monitoring of cancer therapy). The following are the specific aims of the project: (i) develop on-chip cell-lysing approaches from breast aspirates; (ii) develop novel techniques to functionalize the sensor array surfaces with antibodies, while minimizing non-specific adsorption and bio-fouling; (iii) develop novel computational and simulation strategies for modeling the field effect sensor response upon protein binding in fluids, (iv) develop robust top-down silicon-based field-effect nano-plate arrays for multiplexed detection of cancer proteins and markers, and (v) develop integrated biochip sensors and perform extensive tests and preclinical studies. We will keep the focus on the issues and requirements towards assay development to perform ex-vivo preclinical studies with human samples by the end of the project.

258 Molecular Analysis Using Liquid Crystal Technology

Paul J. Bertics[1], Nicholas L. Abbott[2]

Departments of [1]Biomolecular Chemistry and [2]Chemical and Biological Engineering, University of Wisconsin, Madison

The epidermal growth factor receptor (EGFR) is often overexpressed or mutated in human cancers, including non-small cell lung cancers and glioblastomas, and many studies have used the EGFR as a target for the development of cancer therapeutics. However, a lack of understanding about how these agents affect EGFR-mediated events in human tumors, or which EGFR mutations (or over-expression) are most clinically relevant, has made development of these drugs challenging. In this regard, we have established a new class of highly sensitive tools that use nanostructured surfaces and liquid crystals (LCs) to amplify and image molecular interactions so as to assess EGFR expression, phosphorylation, and mutation in cancer cell extracts. This approach, termed the torque balance method, involves measuring a change in the orientation of an LC at a surface in response to an applied torque. Changes in the anchoring energy of the LC in the presence of captured biomolecules (e.g., via their direct surface binding or via their binding to a capture molecule immobilized on the surface) can be measured optically. We used this technique to analyze surfaces patterned with biochemical functionalities relevant to the development of surface-based analytical methods and found that the approach exhibits (i) a sensitivity in the range of pg/mm2, (ii) a compatability with automated data acquisition/analyses, allowing mapping of surfaces with a resolution of 10mm x 10mm, (iii) a capability of characterizing chemical transformations on surfaces, and (iv) an ability to quantify protein binding events over four orders of magnitude (10 pM to 100 nM). In these studies, we established methods for EGFR capture to polydimethylsiloxane (PDMS) surfaces presenting covalently immobilized antibodies directed against EGFR and found that we can detect both total EGFR and phosphorylated (activated) EGFR using either purified EGFR or extracts of transfected fibroblasts or epidermoid carcinoma cell lines. The signal-to-noise obtained with PDMS surfaces was 82:1, exceeding that measured with ELISA plates (<48:1), supporting the concept that LC-based approaches can be used to assess EGFR status in complex solutions such as cell lysates. The approach is being adapted for the screening of clinical specimens, with the goal of using the technology to determine which EGFR alterations/mutations are relevant to specific tumor biopsies so as to aid in determining which cases are most likely to respond to EGFR antagonists. Also, this approach should be readily adaptable to other molecular markers of relevance to the detection and treatment of various cancers.

259 Label Free LC-MS/MS Glycoproteomics for Lung Cancer Serum Biomarker Discovery

William L. Bigbee[1,2,3,4], Xuemei Zeng[1], Thomas P. Conrads[1,2,5], Brian L. Hood[1], Mai Sun[1], Roger S. Day[6,7], Joel L. Weissfeld[3,8], Jill M. Siegfried[2,5]

[1]Mass Spectrometry Platform Laboratory, Cancer Biomarkers Facility, [2]Lung and Thoracic Malignancies Program, and [3]Cancer Epidemiology Program, University of Pittsburgh Cancer Institute; Departments of [4]Pathology, [5]Pharmacology and Chemical Biology, and [6]Biomedical Informatics, University of Pittsburgh School of Medicine; [7]Biostatistics Facility, University of Pittsburgh Cancer Institute; [8]Department of Epidemiology, Graduate School of Public Health, Pittsburgh, PA

Lung cancer remains the leading cause of cancer-related death with a 5-year survival rate of less than 15%. Poor survival is largely due to the late stage at which lung cancer is typically diagnosed and the absence of proven imaging and molecular screening modalities for effective early detection. To discover candidate serum protein biomarkers for early detection of lung cancer, we compared the glycoproteomic serum profiles yielding biochemical identification and relative abundance of proteins among 15 lung cancer case pools (8 adenocarcinoma and 7 squamous cell carcinoma), 8 clinical control pools, and 8 matched healthy control pools. Agilent Human 14 Multiple Affinity Removal spin cartridges were used to deplete the top 14 most abundant serum proteins; the remaining serum proteins were then subjected to hydrazide chemistry-based glycoprotein capture and enrichment. Hydrazide resin in situ trypsin digestion was used to release glycoprotein tryptic peptides (non-glycosylated), and peptide-N-glycosidase F (PNGase F) treatment was used to release formerly N-linked glycosylated peptides. Both sets of peptides were then analyzed by nano-LC-ESI-MS/MS using an LTQ-OrbitrapTM for glycopeptides and an LTQ for non-glycosylated tryptic peptides. A total of 7538 proteins were identified from the combined analyses, including 71 differentially abundant formerly N-linked glycopeptides from 43 proteins from the comparison of extracted glycopeptides ion chromatograms ($P<0.02$, Student's t test) and 360 proteins with differential spectral counts from the glycoprotein tryptic peptides. These identified candidate serum biomarker proteins for subsequent verification include previously described acute phase reactant and inflammatory response species including alpha-1-glycoprotein, vitronectin, leucine-rich-α-glycoproteins, and kinnogen-1.

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260 Reliable Genotyping Using Paraffin-Embedded Tissues: Application to Cancer

Molly S. Bray[1], Laura Post[2], Lara M. Bull[3], Myoung H. Kwon[4], Michael Ittmann[5], **Martha P. Mims**[4]

[1]U.S. Department of Agriculture/Agricultural Research Service Children's Nutrition Research Center, Baylor College of Medicine; [2]Field Applications, Illumina, Inc.; [3]The Human Genome Sequencing Center and Departments of [4]Medicine and [5]Pathology, Baylor College of Medicine

Introduction: In genetic epidemiology studies, gathering an adequate number of case and control samples is a time-consuming and costly process. Stored, fixed tissue could be an abundant, rapidly accessible, and cost effective source of DNA for genotyping studies, but investigators have been reluctant to exploit these samples due to concern that the DNA extracted may be suboptimal for genotyping purposes. The purpose of this study was to evaluate methods for DNA extraction and amplification from paraffin-embedded tissues and to compare DNA samples isolated from various sources in high throughput genotyping assays.

Methods: DNA was extracted from 28 matched samples of frozen and formalin-fixed/paraffin-embedded (FFPE) tissue; FFPE DNA was also subjected to whole genome amplification (WGA). All three DNA sample types (frozen, FFPE, FFPE-WGA) were genotyped using the GoldenGate Test Panel from Illumina. A subset of these samples was also evaluated on the Infinium NS-12 (NS12) and CNV370-Quad high density genotyping panels. Clustering strategies, call rates, and reproducibility were compared across the sample types.

Results: In all assays, call rates were significantly higher when samples were clustered by DNA source. Call rates for WGA samples were significantly lower than other sample types independent of clustering strategy. Average call rates were excellent for all sample types for the GoldenGate assay (99.6±0.5%, 98.8±3.5%, 97.7±2.8%, respectively for frozen, FFPE, and FFPE-WGA samples). Call rates for the Infinium assay were somewhat lower, ranging from 84.3 to 94.1% for FFPE samples and from 80.0 to 86.4% for FFPE-WGA samples. Reproducibility frequency exceeded 0.997 for all sample types. Amplification bias, if present in FFPE-WGA samples, did not affect calling accuracy.

Conclusions: In any study, the most precious resource is the patient sample. We have demonstrated that both unamplified and amplified DNA samples from FFPE blocks provide comparable genotypes and call rates in the GoldenGate assay compared to DNA isolated from frozen tissue samples. While call rates were lower for all sample types in the Infinium assays, a substantial amount of genotyping data was still available from these assays.

261 Microfluidic Devices for Analysis of Circulating Tumor Cells

Yan Chen[1,2], Kuo-wei Huang[3], Yeena S. Ng[1], Pei-Yu Chiou[3], **Jiang F. Zhong**[1]

[1]Department of Pathology, Keck School of Medicine, University of Southern California; [2]Electrical Engineering, Princeton University, Princeton, NJ; [3]Department of Mechanical and Aerospace Engineering, University of California, Los Angeles

With microfluidic devices, biochemical analysis of single cells, which are only a few picoliters, can be performed in a small volume to reduce material loss. We have developed microfluidic devices to perform mRNA-to-cDNA conversion in 10-nanoliter reactors. Our devices can simultaneously process 32 single cells with 5-fold higher efficiency compared to bulk assay. However, the capture and manipulation of single cells into individual reactors precisely require a complex microfluidic network.

Manipulating single cells via controlling fluid flow inside microfluidic devices is complex and inefficient, especially when manipulating a large number of cells. To address these issues for enabling large scale single-cell analysis, we have constructed devices with individual addressable reactors and integrated Optoelectronic Tweezers (OET) technology. These new features enable precise and non-contact manipulation of a large number of live single cells. It not only significantly reduces the complexity of our microfluidic devices, but also significantly improves the simultaneous processing capacity. With these new devices, a population of circulating tumor cells (CTC), isolated from patient blood, can be analyzed simultaneously for various assays inside a single integrated device.

262 Matching Preanalytical Tissue Processing to Testing Platform for KRAS Mutation in Colon Cancer

Wilbur A. Franklin, Jerry Haney, Michio Sugita, Lynn Bemis, Antonio Jimeno, Wells A. Messersmith

University of Colorado, Denver, Aurora, CO

Application of molecular biology advances to a clinical setting requires adaptation of testing platforms to accessible clinical samples. The most widely available samples from solid tumors are formalin fixed-paraffin embedded (FFPE) tissue blocks. In recent clinical trials, treatment of colon carcinoma with the anti-EGFR antibody, Cetuximab, has been ineffective in KRAS mutant tumors. Mutation testing techniques have, therefore, become an urgent concern. To assess the effect of FFPE block sampling on the sensitivity of KRAS testing results, we evaluated the effect on assay sensitivity and specificity of sectioning and coring of paraffin blocks to enrich for tumor DNA in 59 cases of colon carcinoma. We also compared three testing methods for detecting mutations of KRAS including (1) high resolution melting (HRM), (2) amplification refractory mutation system using a bifunctional self probing primer (ARMS/Scorpion, ARMS/S), and (3) direct sequencing. The most sensitive and specific combination of block sampling and mutational analysis was ARMS/S testing performed on DNA derived from 1 mm paraffin cores. This combination of tissue sampling and testing method detected KRAS mutations in 46% of colon tumors. Four samples were positive by ARMS/S but initially negative by direct sequencing. Cloned DNA samples were retested by direct sequencing, and in all four cases KRAS mutations were identified in the DNA at a concentration of 4 to 8%. The high sensitivity of ARMS/S compensated for contamination of tumor DNA with normal DNA template. This study illustrates the necessity of matching preanalytic processing methods with testing platforms to maximize mutation test sensitivity and emphasizes variables that must be taken into account as new molecular information is applied at the bedside.

263 Phase I Trials of Anti-Cancer Agents: The Ohio State University

Michael R. Grever, Catherine R. Balint, Kristie A. Blum, William Blum, John C. Byrd, William E. Carson, Kenneth K. Chan, Ching-Shih Chen, David Cohn, Beth Fischer, Amy J. Johnson, Cheryl Kefauver, Michael V. Knopp, Gregory B. Lesinski, David M. Lucas, David O'Malley, Guido Marcucci, Gregory A. Otterson, Michael A. Phelps, Bhuvaneswari Ramaswamy, Larry J. Schaaf, Manisha H. Shah, Charles L. Shapiro, James Thomas, Miguel A. Villalona-Calero, Deidre Wilkins

The Ohio State University

Phase I clinical trials represent the initial investigation of exciting new therapeutic agents and strategies in patients with cancer. The interface between basic and clinical science is designed to evaluate toxicity in man. In addition to defining the safe dose and schedule of administration, laboratory correlative studies evaluate whether the agent modified the proposed target within the patient's tumor or normal tissues. Furthermore, pharmacokinetics and pharmacogenomics studies are correlated with toxicity and preliminary assessment of efficacy associated with the therapy.

The current emphasis in the era of targeted therapy on defining the in vivo effect of these new agents on target modulation requires sound methodologies as well as careful planning and judgment. The assays to quantitate the impact of the agent on the target must be carefully validated before being applied to patients. Identification of the optimal conditions for sampling and tissue processing must be known before exposing patients to invasive procedures. A careful assessment of the knowledge to be gained versus the risk must be incorporated into the clinical trial. Mechanisms of action of new agents and resistance can be defined by these studies and thus have the potential to aid drug development. If these studies are to be useful in decision-making, the results must be obtainable in near real time. The resources at The Ohio State University dedicated to the early trials in man include creation of a specific phase I oncology unit and a pharmacoanalytic shared resource to facilitate intense monitoring of patients and acquisition of real-time PK and pharmacodynamic data.

Novel agents including small molecules either synthesized by chemists or isolated from natural products, monoclonal antibodies, anti-sense agents, viral oncolytics, vaccines, and cytokines have been studied. While 10 phase I studies are currently open in a variety of patients with either solid tumors or hematological malignancies, 14 new studies are either under consideration by National Cancer Institute or under development at OSU. Novel studies are being specifically developed to address the cancers of burden to under-represented minorities. Several of the existing studies revealed marked clinical benefit for patients with advanced malignancies.

The goal of the OSU Phase I program is to perform rational, efficient, and thorough clinical trials that will generate safe and effective new therapies to benefit cancer patients. Collaboration with basic scientists throughout this process – hypothesis generation, design, implementation, and correlative investigation – greatly enhances the value of each trial.

264 Molecular Mapping of Tumor Heterogeneity With Multicolor Semiconductor Nanocrystals

Jian Liu[1], Stephen K. Lau[2], Vijay A. Varma[2], Richard A. Moffitt[3], Matthew Caldwell[3], Tao Liu[1], Andrew N. Young[1], John A. Petros[1,2], Tracey Krogstad[1], Brian Leyland-Jones[1], May D. Wang[3], **Shuming Nie**[1]

[1]Emory University; [2]Atlanta Veteran Affairs Medical Center; [3]Georgia Institute of Technology

Tumor heterogeneity is one of the most important and challenging problems not only in studying the mechanisms of cancer development but also in developing therapeutics to eradicate cancer cells. Here we report early clinical translation results in using multiplexed quantum dots (QDs) and wavelength-resolved spectral imaging for high-throughput mapping of molecular, cellular, and glandular heterogeneities on human radical prostatectomy tissue specimens. By using just four protein biomarkers (E-cadherin, high-molecular-weight cytokeratin, p63, and alpha methylacyl CoA racemase), we have detected and characterized individual tumor cells within the heterogeneous microenviroments of formalin-fixed paraffin-embedded (FFPE) surgical specimens. Complex architectural changes are associated with cancer development and progression, including prostate glands undergoing structural transitions from a double layer of basal and luminal cells to a single layer of malignant cells. This work is a major step in translating nanotechnology to clinical medicine, raising new possibilities in correlating tissue morphology and molecular biomarker information for cancer diagnosis and treatment selection.

265 The Incidence and Prognostic Significance of Circulating Tumor Cells in Non-Small Cell Lung Cancer

Dena Marrinucci[1], Madelyn Luttgen[1], Daniel Lazar[1], Michael Malchiodi[1], Peter Clark[1], Edward Huynh[1], Kelly Bethel[1], Lyudmila Bazhenova[2], Richard Bruce[3], Peter Kuhn[1], **Jorge Nieva**[4]

[1]The Scripps Research Institute; [2]University of California, San Diego; [3]Palo Alto Research Center; [4]Billings Clinic

Background: While circulating tumor cells (CTCs) have shown promise in breast, prostate, and colorectal cancer, the currently available immunomagnetic enrichment-based technologies have failed to identify these cells in a predictable fashion in lung cancer patients. We have developed a method to identify and image CTCs from non-small cell lung cancer (NSCLC) patients using enrichment and Ep-Cam independent techniques. **Methods:** All patients had metastatic NSCLC with evidence of hematogenously seeded metastasis. All patients had measureable disease. Using fiber array scanning cytometry (FASC), mononuclear cell preparations were immunofluorescently labeled with a mixture of antibodies against cytokeratin and DAPI. Using FASC, these cells were located and imaged using high resolution microscopy. **Results:** Sixty-five patients have been enrolled, with 46 patients having follow-up data available at 3 months. Seven squamous and 39 non-squamous tumors were evaluated. Some126 of 136 patient samples (93%) had CTCs identified. Twenty-five of 46 patients are deceased. At enrollment, the median CTC count was 33cells/8mL. The median CTC count at enrollment for patients who responded or had stable disease at 3 months was 19cells/8mL, while those who progressed or died at 3 months had a median CTC count of 181 cells/8mL. A reduction in the CTC count at the 3-month timepoint was seen in 8/13 patients who responded radiographically at the 3 or 6 month timepoints. There was no reduction in the CTC count at 3 weeks in these responders. Six of seven patients with progressive disease at 3 months demonstrated an increase in the CTC count. **Conclusions:** CTCs can be effectively enumerated in metastatic NSCLC patients, with the majority demonstrating CTCs in the setting of progressive disease. The baseline CTC count carries prognostic significance. The change in CTC count at 3 months but not at 3 weeks correlates with radiographic response to chemotherapy. Further followup will determine the predictive value of CTC enumeration on survival.

266 Basic Science to Clinical Translation: Cancer Programs at the Jackson Laboratory

Kevin D. Mills, Kyuson Yun

The Jackson Laboratory, Bar Harbor, ME

The Jackson Laboratory Program in Cancer Research is a broad, integrated cancer research program that includes research, resource, and services components. Translational cancer research programs at The Jackson Laboratory include studies of lung, brain, and hematologic malignanices. Here we highlight two collaborative, translational cancer research initiatives at the Jackson Laboratory (JAX).

Leukemia: Acute myelogenous leukemia (AML) is a common and heterogeneous hematopoietic malignancy that is fatal for most patients. Despite progress in identifying molecular prognostics to better guide AML therapy decisions, karyotype is still the best AML prognostic marker. Unfortunately, almost one-half of all new AML cases have a normal blast karyotype, thus limiting useful indicators to guide treatment. Thus there is an urgent need to (1) identify new molecular AML markers for clinical decision making and (2) intelligently design individually risk-adapted therapies. Toward these ends, we have initiated a translational leukemia research program in partnership with Memorial Sloan-Kettering Cancer Center (MSKCC), to combine in vitro drug and combination therapy testing success at MSKCC with new advances in the development of humanized mouse models at JAX. In the current collaboration, Dr. Leonard Shultz is developing and validating next-generation mouse models to better support a wide range of human cell xenografts; Dr. Mark Frattini is conducting in vitro and in vivo therapy testing based on individual patient responses; and Dr. Kevin Mills is using humanized mouse models to identify new molecular and genetic markers for risk-adapted clinical management of AML

Glioblastoma: Glioblastoma is a common and aggressive primary brain tumor. We previously identified a 45-gene signature that distinguishes murine glioblastoma stem cells from other cancer and non-cancer cell types. To translate our findings, we are taking three approaches: (1) we developed a sandwich ELISA assay to measure serum levels of a key protein associated with metastasis and high-grade tumors; (2) we are testing whether signature genes can identify human glioma stem cells, in vitro and in vivo; and (3) we are assessing whether these genes are important in glioma stem cell maintenance by knocking down their expression in primary human GBM stem cell cultures. Our findings suggest that the genes we have identified are both biomarkers and molecular targets unique to cancer stem cells in human tumors.

267 Signaling and Progression in Prostate Cancer

Bryce Paschal, **Dan Theodorescu**

University of Virginia, Charlottesville

Metastatic, hormone independent prostate cancer (CaP) is incurable. The goal of this multidisciplinary program project is to elucidate the signal transduction mechanisms that underlie the stepwise events associated with progression of CaP from a localized and androgen sensitive tumor to a disseminated and androgen independent one. The program brings together productive and experienced investigators with complementary expertise relevant to the stated goal of the program and backgrounds in signal transduction (J. T. Parsons, S. J. Parsons, Weber), nuclear receptor biology (Paschal), bone biology (Guise), human prostate cancer pathology (Frierson), biostatistics (Conaway), and basic and clinical prostate cancer metastasis research (Theodorescu). In Project 1, Theodorescu and J. T. Parsons propose to evaluate the roles of VEGF, FAK, and Rap in CaP progression and metastasis to bone. In Project 2, S. J. Parsons studies the regulation of neuroendocrine cell growth within advanced prostate cancers and the impact of such cells on overall tumor dependence on androgen. In Project 3, M. Weber studies Ras-mediated signaling cascades as they affect ligand hypersensitive androgen receptor activity. In Project 4, Paschal proposes to study the relationship between androgen receptor activation and the control of its nuclear localization. This interactive program relies heavily on synergistic technical and scientific expertise from all investigators. The productivity of individual projects is catalyzed by highly interactive cores. Led by Theodorescu, Administrative Core A integrates the participation of M. Conaway, an expert biostatistician. Core B, Cell, Animal and Imaging, is led by Guise, who has extensive experience in bone histology and histomorphometry and is familiar with the biology of prostate cancer and the xenograft models used in prostate cancer research as well as their in vivo imaging. Frierson, an expert surgical pathologist who specializes in CaP, leads Tissue Analysis Core C. Together, these projects and cores integrate diverse skills and expertise to focus on areas fundamental to our understanding of tumor progression in CaP, with the objective of accelerating progress in developing a cure for this devastating disease.

268 Pre-Analytic Assessment of Formalin-Fixed Paraffin-Embedded Tissue Samples for mRNA Transcript Microarray Analysis

Patcharin Pramoonjago, Paul D. Williams, Jae K. Lee, Dan Theodorescu, **Christopher A. Moskaluk**

University of Virginia, Charlottesville, VA

A major bottleneck in translational cancer research is the availability of human cancer tissue for molecular analysis. Frozen tissue samples are usually only procured prospectively in academic medical centers, leaving the majority of available clinical tissue specimens in formalin-fixed paraffin-embedded (FFPE) form, which are suboptimal for many molecular analyses. However, recent reports of advances in RNA isolation and probe labeling have increased the promise of the use of formalin-fixed paraffin-embedded (FFPE) samples in RNA transcript microarray analysis. We have found that a modification of the Qiagen RNeasy FFPE RNA isolation protocol delivers the best combination of RNA quality, RNA yield, and utilization of technician time. Using this method, we isolated RNA from 16 FFPE samples representing a variety of human tumor types and compared the results of Affymetrix GeneChip analysis following isothermal cDNA amplification as deployed in the NuGEN WT-Ovation kit, with the results from RNA obtained from matched flash frozen tissue samples. Some 62.5% (10/16) of FFPE specimens yielded RNA of sufficient quality to produce sufficient labeled cDNA (> 5ug) for microarray analysis. The median correlation coefficient between FFPE and frozen sample microarray analysis was 0.75 (range 0.38 – 0.87), with 30% (3/10) of FFPE samples yielding correlation with their matched sample that was deemed as poor (<0.6). Since both probe amplification/labeling and microarray procedures are costly and time-consuming, we investigated six different qRT-PCR assays of housekeeping genes for their utility as pre-analytic screens of RNA samples. The Ct values for each assay were examined for their correlation with sample performance in these procedures, to determine if clear cut-points could be discerned for sample adequacy. We have found that a 106 bp RT-PCR assay for the beta-2-microglobulin transcript shows promise in identifying FFPE RNA samples that yield microarray gene expression profiles that correlate with results from unprocessed tissue samples.

Our results suggest that approximately one-half of FFPE tissue samples yield RNA of sufficient quality for microarray gene expression profiling that reflects the status of the pre-processed tissue sample. The use of a simple qRT-PCR assay as a pre-analytic assessment is useful in identifying those FFPE samples amenable to microarray transcript profiling.

269 The Pediatric Division of the Cooperative Human Tissue Network

Nilsa C. Ramirez, Jay Bowen

The Research Institute at Nationwide Children's Hospital, Columbus, OH

The Cooperative Human Tissue Network (CHTN) is a group of six academic institutions funded by the National Cancer Institute (NCI) to work together to provide remnant human tissue to researchers throughout the United States and Canada. The CHTN provides normal, malignant, benign, and diseased tissue from routine surgical resections or autopsies. The CHTN is not a tissue bank but prospectively procures and distributes tissues that are not linked to clinical trials. The Pediatric Division of the CHTN (pCHTN) is housed at the Biopathology Center, part of the Research Institute at Nationwide Children’s Hospital in Columbus, OH. The Children's Oncology Group (COG) and the pCHTN have a unique relationship that ensures the proper collection, storage, and availability of high quality, well annotated human specimens, collected from pediatric patient populations and entered into NCI-funded clinical treatment trials. In addition to the traditional CHTN way of prospective tissue procurement and distribution, COG Tissue Bank specimens are distributed via the pCHTN mechanism following evaluation of the scientific value of the request and subsequent approval by the proper COG Disease Committee. Access to the CHTN is provided to any investigator who signs the agreements regarding biohazards and commercial use and who provides a summary of the project for which the tissue is requested. Patient identity or other identifying information cannot be provided to investigators to ensure complete confidentiality of patient medical information. A copy of an approval of the research from the investigator's local institutional review board (IRB or human use) is also required for all projects. The CHTN seeks to provide tissues to the widest group of investigators practicable and attempts to provide each investigator with as many specimens as equitable. Access to specimens varies according to the surgical schedules and autopsy rates and is thus not predictable. A fee for service per sample applies in all cases. A sample is defined as one processed piece of specimen, regardless of the sample size or type of processing. Additional specimen testing (including nucleic acid extractions and tissue macrodissection) can also be performed for a fee. Tissue microarray slides are also available for 23 pediatric cancers and for 5 adult cancers with 2 more in progress. Investigators are obligated to acknowledge the CHTN in any publications that result from their use of specimens received through the CHTN.

270 Isolation and Depletion of Tumor Biomarkers Using Gas Microbubbles [WITHDRAWN]

D. Simberg, R. Mattrey

University of California, San Diego

During cancer disease, malignant cells are shed into blood (1,2). These extremely rare cells (few cells/ml blood) could be isolated and analyzed to provide invaluable information for diagnosis and prognosis of cancer patients (1). Because of low concentration in blood, the isolation of circulating tumor cells (CTCs) is a laborious and expensive process. Immuno-magnetic microbeads are currently used for CTC isolation from blood samples of cancer patients (3). This method is very sensitive (1 CTC/ml blood or lower) but produces significant contamination of non-specific cells in the isolated sample (4) and is practically limited to volumes of less than 10 ml of blood. In order to address the existing problems of CTC isolation from blood, we propose to develop a cell isolation technique based on capture of rare CTCs in blood by gas-filled microbubbles. Targeted microbubbles will find and attach to their target cells in blood, rendering them buoyant and easily separated from all other cells using gravity/centrifugation (Scheme 1). We previously demonstrated (5) that microbubbles coated with anti-FITC antibody were able to specifically bind and deplete FITC-labeled erythrocytes in whole blood in vitro and in vivo. Similarly, microbubbles coated with rituximab (anti-CD20) antibody were able to bind and separate B-cell lymphoma cells from blood. The goal of the first phase of this proposal is to test whether microbubbles could achieve the same efficiency as immunomagnetic bead isolation (1 cell/ml with >90% efficiency), but with greater specificity, speed, and efficiency. Development of a simple, fast, and efficient CTC isolation technology could potentially advance the value of CTCs as an independent diagnostic parameter in cancer medicine.

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271 Sensitive Location and Characterization of Circulating Tumor Cells for Improving Therapy Selection

George Somlo[1], Ben Hsieh[2], Paul Frankel[1], Sean Lau[1], Douglas Curry[2], Robert Krivacic[2], **Richard Bruce**[2]

[1]City of Hope Cancer Center; [2]Palo Alto Research Center

For metastatic disease, biomarker profiling of distant metastases is done only when feasible because biopsy of metastases is invasive and associated with potential morbidity without proven benefit. So, although biomarker expression may differ in distant metastases, treatment with targeted therapies is almost always based on biomarker targets derived from a patient's primary breast tumor, usually excised years before development of metastatic disease.

We have developed a sensitive CTC detection tool using Fiber Array Scanning Technology (FAST) that can rapidly locate CTCs on a substrate and uses abundant cytokeratins, not EpCAM, as targets. We have developed an assay that enables testing for protein expression of four markers in addition to the ones needed for CTC identification. We are currently characterizing locally advanced and metastatic breast cancer patients for expression levels of HER2, ER, ERCC1, and EGFR in CTC. We are using a scoring methodology to provide a numerical score of the sample that is similar to the methodology used in tissue characterization.

We have observed high discordance rates between CTC and tissue characterization. We are validating this discordance to justify incorporating CTC expression into personalized treatment strategies.

Rationale: The modality being developed is "the use of FAST to locate CTCs for characterization to improve treatment selection." FAST is an image-based assessment tool, and its basic technical characteristics have already been validated/credentialed. This fits the "creation of modality" step best because it is being tested in several retrospective cohorts for improvement of treatment selection.

272 Quantum Dots-Based Methods for Highly Sensitive Detection of Cancer Markers

Jeff T.H. Wang

Departments of Mechanical Engineering and Biomedical Engineering, Johns Hopkins University, Baltimore, MD

The unique photophysical properties of semiconductor quantum dots (QDs) have made them ideal for use as spectral labels and luminescent probes. In recent years, there have been several QD applications that utilize these nanocrystals as scaffolds and active participants in biosensing, wherein biological specificity within hybrid inorganic/organic assemblies results in capture and detection of molecular disease markers. The high surface area to volume ratio and well-documented conjugation chemistries for QDs allow attachment of biomolecular probes, thus transforming the nanocrsytals into scaffolds for molecular interactions. QDs also make excellent donors to pair with organic dyes in the fluorescence resonance energy transfer (FRET) process, due to the features of narrow emission spectra and small Stokes shift. This enables FRET with minimal direct acceptor excitation and donor-acceptor crosstalk, thereby permitting the design of FRET molecular sensors with extremely low intrinsic fluorescence backgrounds necessary for detecting biomolecular targets at low abundance. We have demonstrated the use of QDs in developing molecular assays for detecting biomarkers at both the genetic and epigenetic levels. A point mutation assay is developed by incorporating QDs into DNA ligation reactions, facilitating highly sensitive and specific mutation detection in a simplified homogeneous format. This mutation nanoassay has been exemplified with detection of Kras point mutations in clinical samples from patients with ovarian serous borderline tumors (SBTs). In addition, a DNA methylation assay called MS-qFRET is developed based on the above QD-FRET technique. This approach detects as little as 15 pg of methylated DNA in the presence of a 10,000-fold excess of unmethylated alleles and allows for multiplexed analyses. The high sensitivity of MS-qFRET enables one-step detection of methylation at ASC/TMS-1 gene in patient sputum samples that contain low concentrations of methylated DNA, which normally would require a nested PCR approach. The direct application of QD nanoassays on clinical samples offers great promise for its translational use in early cancer diagnosis, prognostic assessment of tumor behavior, as well as monitoring response to therapeutic agents.

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273 The Role of a Surgeon-Based Cooperative Group and Biospecimen Acquisition for Designing Novel Therapeutic Trials: ACOSOG Z1031

Mark A. Watson[1,4], Matthew J. Ellis[1,4], Elaine Mardis[1,4], Heidi Nelson[2,4], David Ota[3,4]

[1]Washington University School of Medicine, St. Louis, MO; [2]Mayo Clinic College of Medicine, Rochester, MN; [3]Duke Clinical Research Institute; [4]American College of Surgeons Oncology Group

The ACOSOG Central Specimen Bank collects, processes, and distributes biospecimens for translational research in the context of clinical trials. Together with members from other cooperative group banks that comprise the National Cancer Institute (NCI) Cooperative Group Bank (CGB) committee, ACOSOG is harmonizing biospecimen informatics and standardizing operating procedures to more effectively leverage CGB resources. ACOSOG trial Z1031 is one example of how these efforts have been put into practice.

Z1031 is a therapeutic trial comparing three aromatase inhibitors (AI) given for 16 weeks in stage II/III, ER-positive breast cancer patients. The primary objective is to determine whether neoadjuvant anastrozole, exemestane, or letrozole should be chosen for a subsequent trial to compare neoadjuvant AI with chemotherapy. Companion correlative studies include central review of ER, PR, Her2, Ki67, and PAM50 gene expression, prediction of response and node metastasis by gene expression profile, and aCGH, gene sequencing to identify mutations in genes associated with therapy resistance, whole genome sequencing, and proteomics studies. Frozen and fixed needle biopsies as well as peripheral blood are collected from participants prior to treatment and at the time of surgery and shipped in specialized procurement kits to the ACOSOG bank. Biospecimen tracking, QC, and distribution data are maintained in the caBIG®- enabled caTissue system. As part of a CGB-wide effort, biospecimen inventory data are shared via a Web-accessible reporting tool. A specialized informed consent template is used to obtain consent for whole genome sequencing.

As of August 2009, all 375 subjects were successfully enrolled. Pretreatment needle core biopsies were collected on 97% of participants. Z1031 was recently amended to continue enrollment of an additional 140 subjects, from whom a third biopsy (2–4 weeks after initial AI therapy) will be collected. Integral Ki67 analysis in a CLIA-licensed laboratory will be used to prospectively determine which patients will continue AI therapy versus neoadjuvant chemotherapy or surgery. Concurrently, whole genome sequencing of 50 tumors is being performed to identify potential new targets for therapy.

Z1031 is just one example of how next generation biospecimen science, informatics, genomics, and clinical trial design is fostering a new level of innovative, biospecimen-based translational research in the context of NCI Cooperative Group trials.

274 Total Cancer Care: Beyond a Biorepository — A Circle of Discovery and Translation for Cancer Patients

Robert M. Wenham, Timothy Yeatman, William S. Dalton

The Total Cancer Care Consortium, H. Lee Moffitt Cancer Center, Tampa, FL

In 2006, a personalized cancer care initiative called Total Cancer Care (TCC) was designed to collect tumor specimens and clinical data throughout a patient's lifetime with the goal of finding "the right treatment for the right patient, at the right time." Because TCC is a lifetime partnership with the patient that involves the collection of data and specimens for research purposes, a formal protocol and patient consent process was developed and an information technology platform was constructed to provide a robust warehouse for clinical and molecular profiling data. TCC primarily has the following goals: (1) identify the needs of the individual patient; (2) identify markers to predict needs and risks; (3) develop methods of early detection; (4) identify signatures predicting which patients will respond to a given therapy; (5) utilize clinical and molecular matching to improve the performance of clinical trials; and (6) raise the standard of care for all patients by integrating new technologies in an evidence-based approach to maximize benefits and reduce costs.

Key elements to TCC include (1) partnerships for research, data and tissue collection, and cancer care delivery, (2) a tissue bank and data warehouse, and (3) data portals providing information to patients, tools and collaborations to researchers, and decision support to clinicians. To date, over 35,000 cancer patients from Moffitt and 16 affiliate medical centers have been enrolled in the protocol. Additionally; over 15,000 tumor specimens have been collected with approximately 8,000 gene expression files generated. We are currently using data from this effort to embark on clinical trials matching patients using genetic signatures. We believe TCC will facilitate discovery of biomarkers for the identification of high-risk populations, early detection, predictors of response and toxicity, and ultimately the most effective treatment for a given population.

275 Development of a Membrane Microfilter Device for Capture and Characterization of Circulating Tumor Cells (CTC) in Blood

Anthony Williams[1], Henry Lin[2], Siyang Zheng[3], Russell Szmulewitz[4], Walter Stadler[4], Howard Scher[5], Martin Fleisher[5], Jian Liu[6], Shuming Nie[6], Debra Hawes[7], Susan Groshen[7], **Ram Datar**[1], Yu-Chong Tai[8], Richard Cote[1]

[1]University of Miami, Miami, FL; [2]Oak Ridge National Laboratory; [3]Pennsylvania State University; [4]University of Chicago Medical Center; [5]Memorial Sloan-Kettering Cancer Center; [6]Emory University; [7]University of Southern California; [8]California Institute of Technology

We present a novel membrane microfilter device (MFD) to isolate circulating tumor cells (CTCs) in blood by exploiting size differences between tumor and normal blood cells. We evaluated the tumor cell capture sensitivity of the MFD in a model system and compared the MFD with the CellSearch platform (the current gold-standard) for isolation of CTC in blood samples from cancer patients. For the model system, five cultured human cancer cells were directly micro-pipetted into 7.5 ml of whole blood from healthy donors and processed using MFD. The cultured tumor cells comprised bladder cancer (J82 and T24), breast cancer (MCF-7, SK-BR-3 and MDA-MB-231), and prostate cancer (LNCaP). In 29 trials, J82 (known to be typically smaller than other cultured epithelial cancer cells) was used, while the other 29 trials examined capture of tumor cells from a mixture of the six different human cancer cell lines to simulate maximal size heterogeneity. The MFD successfully recovered $\geq$1 tumor cell in 96.5% (28/29) and 93.1% (27/29) trials where five cells from J82 or tumor cell-type mixes were used, respectively. Statistical analyses confirms that the true chance of recovering at least 1 tumor cell when five are seeded from 7.5ml of blood is 95% or greater. We also analyzed 7.5 ml blood samples from 57 metastatic cancer patients [prostate (n=28), colorectal (n=12), breast (n=11), and bladder (n=6)]. While the MFD successfully recovered CTC from blood in 92.9% (53/57) of patients, the CellSearch platform recovered CTC in 45.6% (26/57) in corresponding blood samples. When detected by both methods, greater numbers of CTC were recovered by the MFD in all but five patients. We have successfully developed a quadruplexed, multimarker immunofluorescence (IF) assay that can be performed and evaluated directly on the MFD, simultaneously assessing CD44, CD24, ALDH-1, and Cytokeratin utilizing quantum dots as labels. Our data demonstrate that the sensitivity and efficiency of CTC isolation by the MFD compares favorably to the current gold standard CellSearch platform. Independent of an affinity-based CTC capture, the MFD has transformative potential to provide a cheaper, faster, and better alternative to current approaches to CTC isolation, allowing direct, on-chip cell characterization. Future research will employ MFD to enumerate CTC as an early indicator of therapeutic efficacy, and to biologically characterize CTC to identify therapeutic targets, using on-chip multimarker IHC/IF, FISH, PCR and other techniques.

276 Somatostatin Receptor Subtype 2-Targeted Copper Radiopharmaceuticals for Cancer Imaging and Therapy

Albert J. Chang, Kim Nguyen, Rebecca Andrews, Buck E. Rogers, Carolyn J. Anderson

Mallinckrodt Institute of Radiology, Washington University School of Medicine, Saint Louis, MO

Background: Selective receptor-targeting peptides are an important class of radiopharmaceuticals for imaging and therapy. The goal of this work is to develop 64Cu (T1/2=12.7h; B+ (17.8%); B– (38.4%)) radiolabeled-targeting analogs for imaging and treatment of tumors overexpressing the somatostatin (SST) receptor subtype 2 (SSTr2). 64Cu radiopharmaceuticals have made an impact in the area of PET imaging of cancer and are effective targeted radiotherapeutic agents for cancer. 64Cu-radiolabeled SST analogs have wide clinical implications, as SSTr2 are overexpressed in many cancers.

Methods: We synthesized the SST analogs, CB-TE2A-sst2-ANT (antagonist), CB-TE2A-Y3-TATE (agonist), and DOTA-Y3-TATE (agonist), and labeled them with 64Cu. The antagonist is weakly internalized compared to the agonists, allowing for comparison between surface bound and internalized agents. HCT-116 cell lines were transfected with pcHASSTR2 containing the SSTr2 cDNA. HCT-116 cells were utilized, allowing for investigation of p53 as a potential mediator in 64Cu radiotherapy. Receptor binding studies were performed with CB-TE2A-sst2-ANT, CB-TE2A-Y3-TATE, and DOTA-Y3-TATE. Internalization studies were performed with DOTA-Y3-TATE. The MTS proliferation assay was performed to evaluate the effect of DOTA-Y3-TATE on proliferation. In vivo imaging studies are in progress to evaluate the utility of the SST analogs for detection of SSTr2 overexpressing tumors.

Results: HCT 116 cells expressed high levels of SSTr2 after stable transfection and selection. Receptor binding studies demonstrated that the labeled SST analogs, 64Cu-CB-TE2A-sst2-ANT, 64Cu-CB-TE2A-Y3-TATE, and 64Cu-DOTA-Y3-TATE, bound with high affinity with a Kd of 10.49, 0.431, and 1.516 nM, respectively, and Bmax of 37999, 6852, and 3846 fmol/mg, respectively. Internalization studies with 64Cu-DOTA-Y3-TATE demonstrated a time-dependent increase in cellular internalization, which was blocked by unlabeled DOTA-Y3-TATE. MTS assays demonstrated a decrease in cell proliferation with 64Cu-DOTA-Y3-TATE.

Conclusion: 64Cu-labeled SST analogs were successfully synthesized and have high binding affinity to the SSTr2 receptor. Preliminary results demonstrate that these 64Cu-labeled analogs may serve as targeted radiotherapeutic agents for treatment of SSTr2-overexpressing tumors. Furthermore, internalization of these agents may play a role in their therapeutic efficacy. Future studies will be performed to further evaluate their role in imaging and therapy of cancers overexpressing SSTr2.

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277 Real-Time Singlet Oxygen Monitor for Photodynamic Therapy

Steven J. Davis[1], Seonkyung Lee[2], Tayyaba Hasan[2]

[1]Physical Sciences, Inc.; [2]Massachusetts General Hospital

Photodynamic therapy (PDT) is a relatively new, rapidly developing, and promising modality for cancer treatment. PDT uses certain compounds known as photosensitizers (PSs) that are preferentially retained in malignant tumors. With visible light, the photosensitizers initiate a reaction that selectively kills the malignant cells to which they are attached. PDT is being used in clinical trials for bladder, brain, skin, and other cancers. PDT is also being applied to important areas outside of cancer treatment including age-related macular degeneration and actinic keratosis, a pre-cancerous skin condition. There is considerable evidence that singlet molecular oxygen (O2(a1Δ) produced by energy transfer from optically excited photosensitizers (PS) is the active species in cancer cell or endothelial cell necrosis. Despite the general acceptance of this role of O2(a1Δ) in PDT, there have been limited demonstrations of its importance in vivo. If (O2(a1Δ) is indeed the critical species that determines PDT efficacy, a device that is conducive to on-line measurement of O2(a1Δ) in vivo could, in principle, provide the critical parameter in PDT dosimetry and the potential of individualized therapeutic design.

In this poster we describe the development and testing of instruments to measure singlet molecular oxygen produced by the photodynamic process. Singlet oxygen is an active species in photodynamic therapy, and we are developing instruments for PDT researchers with the goal of a real-time dosimeter for singlet oxygen. Our optically-based method uses the weak but unique spectral signature from the O2(a – X) phosphorescence at 1.27 μm. Results of in vitro tests to characterize the sensors and preliminary in vivo results will be presented.

278 Utility of Functional Imaging in Prediction or Assessment of Treatment Response and Prognosis Following Thermotherapy

Mark W. Dewhirst[1], Donald E. Thrall[2], Zeljko Vujaskovic[1], H. Cecil Charles[3], James MacFall[3], Terence Wong[3]

[1]Deparment of Radiation Oncology, Duke University Medical Center; [2]Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC; [3]Department of Radiology, Duke University Medical Center, Durham, NC

Over the past 15 years we have conducted several clinical trials in which we examined the roles that functional imaging (MRI and 31-P MRS) might play in prediction of treatment response and determination of overall prognosis in human and canine patients with soft tissue sarcomas or women with locally advanced breast cancer (LABC) who were enrolled in thermotherapy trials, either in combination with radiotherapy, chemotherapy, or both. We utilized traditional outcome parameters: local response, local control and progression free or overall survival for comparison with imaging parameters.

Changes in ATP/Pi after the first heat treatment were correlated with response rate but not with other outcome variables in soft tissue sarcomas. Additionally, DCE-MRI perfusion patterns were strongly associated with clinical response in LABC.

Of more interest, perhaps, were the imaging parameters that correlated with metastasis free and overall survival. Parameters that were strongly associated with these more important outcome variables in soft tissue sarcomas of both humans and dogs included phoshomonoester / phosphodiester (PME/PDE) (3, 4), extracellular pH (4) and DCE/MRI parameters related to washout rate (5). In humans, the soft tissue sarcomas were all high grade, and approximately 50% of such patients eventually develop metastases, although there is currently no method to predict this a priori. These parameters might be of important value in predicting who is at high risk for development of metastases in soft tissue sarcoma.

279 Self-Assembled "Mothership" Nanoparticles for Integrated Cancer Imaging and Therapy

Hongwei Duan, **Shuming Nie**, Lily Yang, Dong Shin

Emory University

We report two cancer theranostic nanoparticles based on a new class of self-assembled “mothership” nanostructures. These nanoparticle agents are designed to first “navigate” the body’s vascular system, “dock” at the perivascular regions, and then to “unload” or dissociate into small cargos for efficient delivery to the tumor microenvironments and tumor cells. In comparison with current nanoparticles such as liposomes, dendrimers, and PLGA, our theranostic nanoparticles utilize an entirely new in vivo delivery platform based on hyperbranched polyglycerols (PG). This in vivo delivery platform has a large number of peripheral functional groups (hydroxyls) for direct conjugation to both imaging and therapeutic agents and is able to rapidly clear from major reticulo-endothelial (RES) organs such as the liver and spleen. In fact, branched and neutral PGs are so biocompatible and nontoxic that they have been considered a type of “synthetic albumins” for use as osmotic blood expander at high concentrations. Recent work has shown that branched PGs are able to self-assemble into 50– 80 nm particles when conjugated to cancer drugs and targeting ligands, and that these self-assembled nanoparticles are able to dissociate into smaller pieces in the presence of blood proteins. This has led to a new class of theranostic nanoparticles for integrated cancer imaging and therapy, with unusual tumor accumulation and RES evading properties. Toward clinical translation, we have recently examined the blood circulation, in vivo targeting of solid tumors, biodistribution and anticancer activity of these self-assembled and highly biocompatible nanoparticles in animal models. The self-assembled nanoparticles have shown excellent tumor targeting though passive targeting and ligand-directed active targeting while maintaining low non-specific RES uptake. Our results also show that linking an optical imaging probe (i.e., a near-infrared dye, cy5.5) enables the in vivo imaging and tracking of the delivery of nanoparticles, providing valuable information for optimization of nanoparticles by directly monitoring the nanoparticle pharmacokinetics and biodistribution.

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280 Molecular Imaging of Cancer and Its Response to Therapy

Janet Eary[1,2], **Kenneth Krohn**[1,2], Jeanne Link[1,2], David Mankoff[2,3], Mark Muzi[1], Finbarr O'Sullivan[4], Alexander Spence[1,2]

[1]University of Washington, Seattle, WA; [2]Fred Hutchinson Cancer Research Center, Seattle, WA; [3]Seattle Cancer Care Alliance, Seattle, WA; [4] University College, Cork, Ireland

Our program develops and evaluates complementary PET radiopharmaceuticals and data analysis methods to quantify molecular differences between tumors and normal tissues. Understanding important molecular differences and how they change during treatment should lead to better characterization of tumor biology and ultimately better treatment outcome. Our research tests this hypothesis for predicting response to specific treatments and how images change over the course of treatment. Radiopharmaceuticals that image specific biochemical processes should have independent significance above that of conventional imaging with FDG PET, CT, and MRI. One of our goals is to test the extent to which specific molecular aspects of the tumor phenotype are common across histologies. For example, characteristics such as growth rate and the cure-limiting effects of hypoxia or acquired multi-drug resistance should be independent of the cell of origin. Imaging is also important for measuring the in vivo heterogeneity of tumors. Studies investigate the following: (1) [11C]-thymidine and [18F]-3'-fluorothymidine are proliferation imaging agents that trace the thymidine salvage pathway. They are being investigated as measures of treatment response and to distinguish treatment effects on normal tissue from tumor progression; for example, in pseudoprogression of glioblastoma in patients receiving temozolominde and radiotherapy. (2) We have developed methods for measuring drug resistance mediated by upregulation of P-glycoprotein using [11C]-verapamil and PET and are testing the hypothesis that patients treated with anthracycline-based regimens with high P-gp activity will have poorer response to therapy and earlier metastases. (3) We have shown that hypoxia imaging using [18F]-FMISO is predictive of outcome in head and neck cancer and glioma. We now test FMISO PET for radiotherapy treatment planning and study changes in hypoxia in response to anti-angiogenic therapy in gliomas and in response to HER2-directed therapy in breast cancer. (4) We are studying breast cancer ER expression by 18F-fluoroestradiol (FES) PET to direct endocrine therapy and evaluate the pharmacodynamics of novel therapy directed at ER expression such as histone deacetylase (HDAC) inhibitors. (5) We are developing and testing new molecular imaging agents for cancer including probes for drug transport, androgen receptor expression, and MAO-A expression in prostate cancer.

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281 Breast Cancer Molecular Profiles Are Predictive of Tumor Response to Neoadjuvant Chemotherapy, The I-SPY TRIAL (ACRIN 6657, CALGB 150007/150012, InterSPORE)

Laura J. Esserman[1], Charles M. Perou[2], Maggie Cheang[2], Angela DeMichele[3], Lisa Carey[2], Laura J. van 't Veer[4], Joe Gray[1], Kathleen Conway[2], Baljit Singh[5], Meredith Buxton[1], Sarah E. Davis[1], Donald Berry[6], Nola Hylton[1], and the I-SPY Investigators

[1]University of California, San Francisco; [2]University of North Carolina at Chapel Hill; [3]University of Pennsylvania, Pittsburgh, PA; [4]Netherlands Cancer Institute, Amsterdam, Netherlands; [5]New York University, New York, NY; [6]M. D. Anderson Cancer Center, Houston, TX

The I-SPY TRIAL is a neoadjuvant multi-center trial designed to identify predictive markers of pathological complete response (pCR) and survival of women with locally advanced breast cancers (>/=3cm). Patients were treated with AC->T. **Methods:** Of 237 patients enrolled, 216 completed serial imaging and core biopsies. Pre-treatment assays included Agilent expression arrays, MIP aCGH, p53 gene chip and sequencing, and IHC. Response to therapy was measured by serial MRI, pCR, and residual cancer burden (RCB), a quantitative histopathologic measure of cancer that identifies a group near pCR (RCB I). Associations among molecular markers, pCR, RCB, and survival were evaluated using chi-square tests, K-M curves, and log-rank test. All data are centrally stored on caINTEGRATOR. **Results:** Median tumor size was 6cm, with 27% pCR and 36% RCB 0/I; 25% pCR for the 144 Agilent arrays. Several molecular subtypes, including NKI 70 gene low, luminal A, and IHC Hormone Receptor+ (HR), define 9–48% of patients with 0–10% pCR, yet excellent early survival, whereas patients with high-risk molecular profiles: NKI 70 gene high (91%), IHC HR– Her2+ (12%) or IHC HR-Her2– (28%), activated wound healing (77%), and basal subtype (32%) define patients with pCR rates of 28–59% to standard chemotherapy. RCB was more predictive of DFS and OS ($p=0.01$) than pCR alone, with a mean followup of 3.9 years. MR volume is highly predictive of pCR and RCB. For poor risk subtypes, RCB is incredibly predictive of DFS ($p<0.001$). **Conclusion:** Patients with good prognosis profiles had few or no recurrences at 3 years in spite of low rates of response to chemotherapy. Yet, patients with high-risk profiles had a good response to chemotherapy, as measured by RCB, is incredibly predictive of recurrence at 3 years. I-SPY TRIAL data support the need to target improvement to pCR/RCB to improve outcomes in poor prognosis patients and provides a platform to compare, contrast and combine marker signatures to tailor therapy. This is the foundation of the I-SPY 2 TRIAL. I-SPY 2, a collaboration of NCI, FDA, and fNIH BC, is a neoadjuvant phase 2 adaptive design trial process to test novel agents in combination with chemotherapy to improve the rate of pCR in women with poor risk profiles. I-SPY 2 will extend the IT infrastructure from I-SPY 1 and eliminate many of the inherent organizational ineffeciencies in clinical trials. Predicted likelihood of success in phase 3 trial and predictive biomarkers will accompany each drug that leaves the trial. I-SPY 2 will be a model for accelerating the pace of identifying and developing molecularly targeted agents and improving outcomes for women with high-risk cancers.

282 Validation of the Pulmonary Metabolic Radiation Response as an Imaging Biomarker

Thomas Guerrero

University of Texas M. D. Anderson Cancer Center

This study seeks to develop the first meaningful objective radiographic parameter that correlates with the clinical symptoms of radiation pneumonitis (RP) as an imaging biomarker of RP. RP is the dose limiting toxicity in thoracic radiotherapy for lung cancer, making local control (based on bronchoscopic biopsy) achievable in less than 20% of patients. RP is an inflammatory reaction within lung tissue in response to radiation injury. [18F]-2-fluoro-2-deoxyglucose positron emission tomography (18F-FDG PET) imaging provides an assessment of pneumonitis, pulmonary inflammation that appears as enhanced 18F-FDG uptake in response to inflammatory stimuli. We found with statistical modeling a linear relationship between the 18F-FDG PET uptake (normalized to unirradiated lung) and the radiation dose in normal lung after radiotherapy for esophagus cancer. We found this linear relation in each patient examined; however, the slope varied from individual to individual over an order of magnitude. We refer to the regression slope of this relationship as the pulmonary metabolic radiation response (PMRR). We found a significant correlation between clinical symptoms and the PMRR measured by 18F-FDG PET imaging following thoracic radiotherapy. Using the PMRR as a surrogate imaging biomarker of toxicity, we found a greater PMRR response among thoracic radiotherapy patients who received both induction and concurrent taxane chemotherapy. The development of the PMRR as a RP imaging biomarker requires a prospective study to demonstrate reproducibility and correlation with clinical symptoms. Our hypothesis is: The PMRR, first described by the principal investigator, provides an imaging biomarker of clinical and molecular pulmonary radiation toxicity. To develop this hypothesis we will conduct a phase II imaging trial to obtain 18F-FDG PET imaging post-radiotherapy in non-small cell lung cancer patients. To improve the clinical correlation compared with our prior retrospective studies we will (1) query respiratory symptoms weekly using a standard respiratory questionnaire and (2) utilize a consistent PET imaging post-radiotherapy delay and post-injection uptake period. We will assess the correlation of radiation induced inflammatory signaling, cytokine, and adhesion molecules with the metabolic response. Weekly measurement of these molecular biomarkers will allow their predictive power to be evaluated and provide for correlation with the PMRR and clinical symptoms. We anticipate the PMRR, the focus of this study, may have a significant impact on the development of drug to reduce radiation treatment toxicity in cancer patients.

283 A Pilot Study for Using 3'-Deoxy-3'-[18F]Fluorothymidine Positron Emission Tomography ([18F]FLT PET/CT) Imaging for Early Assessment of Response to Induction Chemotherapy in Acute Myeloid Leukemia

Mark Juckett, Matt Vanderhoek, Scott Perlman, Robert Nickles, Robert Jeraj

University of Wisconsin, Madison

Background: AML patients treated with induction chemotherapy undergo a bone marrow assessment after 10–14 days to determine the response to therapy, but this is an imperfect predictor of later CR. Thymidine uptake reflects chemotherapy sensitivity in vitro, and we hypothesized that this would also be true in vivo. To test this hypothesis, we performed a pilot study of [18F]FLT PET/CT scans in patients undergoing induction chemotherapy for AML. **Methods:** Seven patients with AML receiving induction chemotherapy underwent whole body PET/CT scans at different time points (pre-, day 1, 3, 4, 5, and 14) using approximately 5 mCi of [18F]FLT. Two patients had scans pre-treatment and day 14. Ten subjects without blood disease also underwent FLT PET/CT as normal controls. The CT images were used to reconstruct a skeletal mask that was used to extract the bone marrow signal from the PET images. Scans from those with normal bone marrow were used for baseline parameters. The images from the scans were quantified by computer analysis into standardized uptake values (SUV). The bone marrow SUV (BM-SUV) were compared between the AML patients relative to normal controls. The mean and max SUV values and coefficient of variation (CV) were used in the comparison. **Results:** Of the 7 patients, 3 entered a complete remission after a single course of induction (CR), 2 required a second induction to achieve CR (PR) and 2 had overtly refractory disease (PD). The responders had recovery of an ANC > 500 by day 22, 28, and 35, suggesting the [18F]FLT did not delay bone marrow recovery. Mean and max BM-SUV by response group are listed in the table:

	SUVmean	SUVmax	CV
CR	0.76 ± 0.04	3.6 ± 0.1	0.29 ± 0.01
PR	0.87 ± 0.01	4.0 ± 1.1	0.37 ± 0.04
PD	1.60 ± 0.14	11.4 ± 0.8	0.71 ± 0.04

The fall in BM-SUV occurred quickly and as early as day 1 after the beginning of chemotherapy among CR and PR patients. The BM-SUV was significantly lower in the CR and PR patients compared to those with PD. The differences in SUV and signal variation in the bone marrow were easily apparent when viewing the images. The heterogeneity in axial skeletal uptake among those with PD was notable and suggests that chemotherapy responsiveness is not uniform throughout the bone marrow and could explain false-negative day 14 bone marrow assessments. **Conclusion:** In appears that FLT PET/CT could be an early predictor of CR among patients undergoing induction therapy for AML.

284 In Vivo Quantification of Changes in HER2 Expression Following Therapeutic Intervention

Gabriela Kramer-Marek[1], Dale O. Kiesewetter[2], **Jacek Capala**[1]

[1]National Cancer Institute and [2]National Institute of Biomedical Imaging and Bioengineering, NIH, Bethesda, MD

In vivo imaging of HER2 expression may allow a direct assessment of HER2 status of primary and metastatic lesions. We have used 18F-ZHER2-Affibody molecule to study the effect of 17-DMAG on HER2 expression in tumor xenografts by PET imaging. To assess the correlation of signal observed by PET with receptor expression, the tracer was administered to athymic nude mice bearing subcutaneous tumors with different levels of HER2 expression: 1. BT474 (very high), 2. Mcf-7/clone18 (high), 3. MDA-MB-361 (medium), 4. Mcf7 (very low), and 5. MDA-MB-468 (negative). Our results showed that 18F-ZHER2-Affibody was eliminated quickly from the blood and normal tissues, providing high tumor/blood and tumor/muscle ratios already 1h post injection. The high contrast images between normal and tumor tissue were recorded for BT474, Mcf7/clone18 tumors. Very low but still detectable uptake was observed for Mcf7 tumors and none for MDA-MB-468. The signal correlated well with the number of receptors expressed in those particular tissue lysates as assessed by western blot, ELISA, and IHC. To study the downregulation of HER2, mice were treated with four doses (50 mg/kg) of 17-DMAG, an inhibitor of Hsp90, known to decrease the HER2 expression. Animals were scanned before and after the treatment. Immediately after the last scan, mice were euthanized, and tumors were frozen for analysis of receptor expression. In animals bearing BT474 tumors, the level of HER2 expression, estimated by PET imaging, decreased 50–60% post-treatment. This change was confirmed by the biodistribution studies, ELISA and western blot. These results indicate that the described 18F-ZHER2-Affibody radioconjugate can be used to assess HER2 expression in vivo by PET imaging and to monitor possible changes of receptor expression in response to therapeutic interventions. Since Affibody molecules do not affect the targeted cells, and their binding does not interfere with either binding or effectives of trastuzumab, we are currently using our probe to study downregulation of HER2 following treatment with trastuzumab and its possible correlation with tumor response as measured by changes in the tumor volume.

285 Positron Emission Tomography (PET) Applications in Phase I Clinical Trials: The Use of 18F-FAU to Identify and Treat Patients With High Thymidylate Synthase Activity

Patricia M. LoRusso, Angelika M. Burger, Lance Heilbrun, Anthony F. Shields

Barbara Ann Karmanos Cancer Institute, Wayne State University, Detroit, MI

Prodrug approaches in cancer drug development are aimed to enhance the tumor selectivity of anticancer agents. 1-(2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl) uracil (FAU, or NSC# 678515) is a suicide prodrug that must be phosphorylated by thymidine kinase and methylated by thymidylate syntheses (TS) in tumor cells before it can be incorporated into their DNA, causing cancer cell-specific death.

TS is an enzyme with an essential role in the synthesis of DNA. It acts by converting deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which allows DNA biosynthesis. TS protein and mRNA levels are elevated in many human cancers, and high TS levels have been correlated with poor prognosis in patients with colorectal, gastric, breast, cervical, kidney, and non-small cell lung cancers. One mechanism by which cells acquire resistance to 5-FU is upregulation of TS expression. It has been shown that tumors with high TS expression are generally nonresponsive to regimens that include the TS-directed combination of 5-FU and leucovorin. Tumors with high levels of thymidylate synthase (TS) represent a common therapeutic challenge for which no specific treatment is currently available. Thus, the rationale for FAU development is to offer a treatment alternative for patients with intrinsically high TS tumor levels or those who no longer respond to TS inhibitors due to an upregulation of TS as a means of acquired drug resistance.

We have recently evaluated 18F-FAU as an alternative positron emission tomography (PET) agent for 11C-thymidine and shown that its uptake rapidly declines after successful therapy in solid tumors such as lung cancer and sarcomas. 18F-FAU may have some advantages over thymidine in that it is labeled with 18F, it is not readily degraded, and may be used to measure a specific step in the DNA synthetic pathway - TS.

The availability of 18F-FAU enabled us to design a phase I clinical trial of nonradioactive FAU as a suicide prodrug with correlative studies emphasizing PET imaging with 18F-FAU to measure incorporation of drug into tumor and normal tissues. In addition, thymidylate synthase and thymidine kinase gene expression and protein levels are measured as response markers.

We will present our experience using 18F-FAU as a PET probe in both dogs and humans, the preclinical data providing the rational for FAU as an antitumor agent and preliminary result of the clinical trial.

286 Molecular Breast Cancer Imaging Research at the University of Washington (UW) and Seattle Cancer Care Alliance (SCCA)

David A. Mankoff, Hannah M. Linden, William E. Barlow, Lisa K. Dunnwald, Robert Doot, Janet F. Eary, V.K. Gadi, Julie R. Gralow, Paul E. Kinahan, Brenda F. Kurland, Connie D. Lehman, Jeanne M. Link, Larry R. MacDonald, Mark Muzi, Savannah C. Partridge, Lanell M. Peterson, Erin K. Schubert, Jennifer M. Specht, Kenneth A. Krohn

University of Washington, Fred Hutchinson Cancer Research Center, and Seattle Cancer Care Alliance, Seattle, WA

Molecular imaging research in breast cancer is a part of a larger multi-disciplinary effort in molecular imaging of cancer at the UW and Fred Hutchinson Cancer Research Center (FHCRC) Cancer Consortium. Research emphasizes molecular imaging as a tool for guiding breast cancer therapy and for quantifying in vivo breast cancer biology. Our work focuses largely on positron emission tomography (PET) but also encompasses functional MRI methods and work in kinetic modeling and quantitative image analysis. Major areas of emphasis include (1) quantifying bone metastasis response to systemic therapy, (2) measuring the response of locally advanced breast cancer to systemic therapy, including targeted systemic therapy, (3) measuring regional estrogen receptor (ER) expression and estradiol binding to guide breast cancer endocrine therapy, (4) identifying in vivo biologic factors associated with response and resistance to systemic therapy, and (5) translating early studies from our center into multi-center trials. Accomplishments arising from these areas of investigation include (1) 18F-fluorodoxyglucose (FDG) PET to measure breast cancer bone metastasis response to therapy, (2) 18F-fluoroestradiol (FES) PET to predict breast cancer response to endocrine therapy and measure the pharmacodynamic effect of endocrine treatments, (3) identification of a tumor flow/metabolism mismatch as a predictor of therapeutic resistance and poor patient outcome, (4) demonstration of tumor hypoxia in a subset of locally advanced breast cancers using 18F –fluoromisonidazole (FMISO) PET, (5) use of 18F-fluoride PET and kinetic analysis to measure regional bone metastasis perfusion and new bone formation simultaneously, (6) quantification of changes in breast cancer perfusion and metabolism in response to targeted therapy, and (7) translation of results and methods into cooperative group imaging trials. These examples highlight the translational capacity of molecular imaging in breast cancer patients and provide examples of how molecular imaging can be used for the study of in vivo cancer biology, to direct cancer clinical trials, and as part of clinical cancer care.

287 Update on RT-Induced Cardiopulmonary Injury

Lawrence B. Marks[1], Jessica L. Hubbs[1], Michael V. Lawrence[1], Donna R. Hollis[2], Junan Zhang[3], Jinli Ma[3], Terence Z. Wong[4], Ronald J. Jaszczak[4], Rodney J. Folz[5], Senem Demirci[3], Michael A. Blazing[6], Sumin Zhou[7], Salvador Borges-Neto[4]

[1]Department of Radiation Oncology, University of North Carolina, Chapel Hill; [2]Departments of [2]Biostatistics, [3]Radiation Oncology, and [4]Radiology, Duke University Medical Center, Durham, NC; [5]Pulmonary, Critical Care, and Sleep Disorders Medicine, University of Louisville Health Science Center, Louisville, KY; [6]Division of Cardiovascular Medicine, Duke University Medical Center, Durham, NC; [7]Department of Radiation Oncology, University of Nebraska Medical Center, Omaha, NE

Goal: To better understand determinants of radiation therapy (RT)-induced cardiopulmonary injury. Better predictors can guide plan modification and/or use of protectors/mitigators. **Methods:** Changes in regional lung/heart perfusion are assessed by pre- and post-RT single photon emission computed tomography (SPECT) scans, providing 3D perfusion maps. Registering SPECTs with RT planning CTs (and 3D doses) allows perfusion changes to be related to regional RT dose, providing dose-response curves (DRCs). The "sum" of regional lung/heart SPECT changes is related to changes in global lung (e.g., pulmonary function tests [PFTs]) and heart function (e.g., ejection fraction). Degree of lung/heart changes is related to changes in global cardiopulmonary function (e.g., symptoms, peak O2 consumption). Recent initiatives include (a) assessing the impact of "low-dose lung bath" (quantified by V5: % lung ≥5Gy) on the sensitivity of the lung to RT (quantified by DRC slope); (b) relating PFT changes to the extent/severity of lung density changes; (c) relating post-RT clinical symptoms to changes in PFTs; and (d) relating changes in regional cardiac perfusion to chest pain. **Results:** (a) In 68 patients, the DRC slope 6 months post-RT is slightly higher for patients with V5s > vs < the mean (0.96 vs 0.75 %/Gy, p=0.02), suggesting possible "neighborhood" effects. Techniques that increase lung volumes exposed to low doses (e.g., intensity modulation) may increase lung sensitivity (Marks Astro 09). (b) In 111 patients with 203 post-RT exams, there were modest correlations between changes in PFTs and the extent/severity of changes in lung density (r=0.20–0.43) (Ma IJROBP 09), supporting the concept of relating regional and global organ injury. (c) In 141 patients, there are no significant links between post-RT shortness of breath and declines in PFTs (p=0.15-0.4) (Hubbs Astro 09); thus, shortness of breath may reflect more than lung injury. (d) With 2–9 years followup, clinical cardiac event rates in patients with vs without RT-associated perfusion defects (at 6–24 months post-RT) were 2/51 and 3/48, respectively (p=0.6). Clinical events correlated with pre-RT cardiac risk factors; thus, relatively short-term post-RT cardiac events appear unrelated to RT-associated perfusion defects. Additional followup is needed to understand if SPECT defects are clinically relevant (Demirci Astro 08).

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288 Clinical Development of an Oncolytic Measles Virus Expressing the Sodium Iodide Symporter (NIS) Imaging Gene

Kah-Whye Peng, Stephen J. Russell

Department of Molecular Medicine, Mayo Clinic, Rochester, MN

Multiple myeloma is an incurable, disseminated malignancy of terminally differentiated plasma cells that is responsible for the deaths of more than 11,000 Americans every year. New approaches to therapy are urgently required. Attenuated measles viruses are selectively destructive to human myeloma plasma cells and are therapeutically potent in murine myeloma xenograft models. In part, this is because myeloma cells express high levels of the measles receptor CD46, which mediates efficient virus entry and subsequent measles-driven intercellular fusion. A recombinant measles virus, MV-NIS, is currently being tested as an intravenous therapy for patients with advanced, treatment-refractory multiple myeloma in an NCI-sponsored phase I clinical trial. Expression of the NIS (sodium iodide symporter) protein in infected cells allows noninvasive radioiodine imaging of sites of viral gene expression via SPECT/CT.

However, antimeasles antibodies represent a potential Achilles heel that may limit the therapeutic efficacy of systemically administered measles viruses. Because myeloma is associated with suppression of humoral immunity, antimeasles antibody titers are significantly lower in these patients than in normal adults, making myeloma an ideal clinical target for systemic oncolytic measles virus therapy. However, at least 70% of myeloma patients do have antibody titers that would be expected to significantly accelerate the destruction of circulating viruses such that their infectivity may be partially or fully neutralized by the time they arrive at the target site. Also, antimeasles antibody titers are expected to increase progressively in myeloma patients, with each exposure to the virus resulting in faster virus inactivation, either by direct neutralization, complement-mediated lysis, or accelerated reticuloendothelial clearance. Thus, we are currently exploring strategies to improve the efficiency of measles delivery to disseminated myeloma sites in measles-immune subjects by using immunosuppressive drugs and/or cells to serve as carriers for MV-NIS.

289 Imaging of GVHD and GVL Using CD34-TK-Modified Allogeneic T Cells

Michael P. Rettig[1], Julie K Ritchey[1], Linda Eissenberg[1], Julie L. Prior[2], Sally Schwarz[3], Vijay Sharma[2], David Piwnica-Worms[2,4], John F. DiPersio[1]

[1]Division of Oncology, [2]Molecular Imaging Center, [3]Division of Radiological Sciences, Mallinckrodt Institute of Radiology, and [4]Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO

Patients with leukemia or lymphoproliferative disorders who receive allogeneic hematopoietic stem cell transplantation (HSCT) but then later relapse have only a 50% survival rate. Donor lymphocyte infusions (DLI) cure some patients, but while the donor T cells begin to mediate the beneficial graft-versus-leukemia (GVL) effect, they can also react against the recipient's tissues, causing life-threatening graft-versus-host disease (GVHD). Currently, no reliable test exists to know whether patients will develop GVHD following transplantation or DLI. Often, the disease is already progressing toward severe organ damage by the time clinical symptoms such as skin rash occur. The ideal scenario in allogeneic HSCT would be to infuse T cells from a matched donor to exert the GVL response but to eliminate them if GVHD begins to develop. Our trial, approved by the Recombinant DNA Advisory Committee (RAC), Food and Drug Administration (FDA), and Institutional Biosafety Committee (IBC), seeks to do this by infusing donor T cells transduced with a chimeric human CD34-HSV1-thymidine kinase (CD34-TK) suicide gene into patients who have relapsed after allogeneic HSCT. The CD34 epitope allows efficient selection of engineered cells, and the TK protein allows the transduced cells to be killed by the prodrug ganciclovir (GCV). In preparation for our clinical trial, we have performed extensive preclinical studies evaluating the efficacy of the CD34-TK/GCV method of GVHD control. First, we developed robust protocols for the production and retroviral vector integration site analysis of CD34-TK-modified human T cells. Second, we demonstrated in a murine transplant model that the CD34-TK/GCV suicide gene therapy system can mitigate GVHD while retaining a GVL effect. Finally, using a xenogeneic model of human T cell-induced GVHD, we showed that the HSV1-TK substrate, [18F]FHBG, and positron emission tomography (PET) could be used to track CD34-TK transduced T cells and predict the onset of xenogeneic GVHD in immunodeficient mice. In an amendment to our pending clinical trial, we have proposed to image CD34-TK-transduced T cells in the recipient with [18F]FHBG and PET. This imaging assessment should allow us to define patterns of T cell trafficking in humans after DLI that are predictive of GVHD before clinical symptoms occur and provide a non-invasive molecular marker to guide the timing of therapeutic GCV administration. Results from this novel T cell suicide gene therapy/ DLI infusion/ PET imaging study could significantly extend the life expectancy of patients with relapsed leukemia after allogeneic transplantation.

290 Mix and Match Nanodendrons for Targeting and Treatment of Breast Cancer Metastases

Randy Scherer[1], Lynn Samuleson[3], Oliver McIntyre[1], Darryl Bornhop[3], Lynn Matrisian[2]

[1]Interdisciplinary Materials Science & Engineering, Vanderbilt University; [2]Department of Cancer Biology, Vanderbilt-Ingram Cancer Center, Nashville, TN; [3]Department of Chemistry, Vanderbilt University, Nashville, TN

The survival benefit of adjuvant therapy for node negative breast cancer, in which an estimated 35% of women have non-detected, micrometastatic disease at the time of first diagnosis, supports the concept that targeting "early stage" metastases can be effective in eliminating death due to metastases. Optical beacons have been successful in imaging proteolytic activity associated with the tumor microenvironment and metastases, and prodrug therapies have been explored to reduce toxic side effects of anti-cancer agents. We are developing "mix-and-match" dendrimeric nanoparticles and testing them for enhanced efficacy in the detection and treatment for breast cancer metastases. Individual components are made on nanodendron (ND) scaffolds (ND1-proteolytic beacon, ND2-prodrug, ND3-apoptosis sensor) that can be combined to form distinct multi-functional nanodendrimers with specific functionality.

ND1-proteolytic beacon is a polyester generation-2 dendron covalently coupled to an AF700-labeled peptide that is a selective substrate to monitor MMP-9 activity (sensor) with AF750 acting as a FRET pair and an internal reference to monitor substrate concentration. Preliminary studies with the substrate show an approximate ~7.5 fold increase in sensor activation upon substrate digestion and a sensor to reference ratio of 10 allowing for effective monitoring of substrate activity.

ND2-prodrug is built on the polyester generation 2 dendron covalently coupled via the same MMP-9 substrate with Paclitaxol (PXL) at the N-terminus. The MMTV-PyVT mouse model of breast cancer will be used to test the sensitivity and efficacy of the NDs individually and in combination. Cell lines derived from PyVT tumors have been treated with ND1 in vitro using the trypan blue assay to assess toxicity. Doses of PXL as low as 50nM elicited an effective toxic response. PXL conjugated to the scaffold had minimal toxicity and upon cleavage by MMP9 toxicity was restored. Furthermore, treating the MMP9 positive cell line with a general MMP inhibitor blocked the toxicity of the PXL-Peptide-Dendron.

Future studies will investigate the effectiveness of mix-and-match combinations of ND1 and ND2 in PyVT-derived cell lines in FVB-WT and MMP9-null mice lacking host MMP9. This model system will evaluate dose response to MMP-9 by optical imaging for the beacon function ND1 and by bioluminescence and histological assessment using a TUNEL assay of tumor cell death for the prodrug therapeutic function ND2.

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291 A Platform of Molecularly Targeted Nanostructures for Anticancer Therapy With Cytolytic Peptides

Neelesh R. Soman, Gregory M. Lanza, John E. Heuser, Jeffrey M. Arbeit, Paul H. Schlesinger, **Samuel A. Wickline**

Washington University, St. Louis, MO

The in vivo application of cytolytic peptides for cancer therapeutics is hampered by toxicity, non-specificity, and degradation. We have developed a specific strategy to synthesize a nanoscale delivery vehicle for cytolytic peptides by incorporating the amphipathic peptide melittin into the outer lipid monolayer of a perfluorocarbon nanoparticle. The favorable pharmacokinetics of this nanocarrier allows higher accumulation of melittin in murine tumors and a dramatic reduction in tumor growth without any apparent signs of toxicity. Alternatively, direct assays demonstrate that molecularly targeted nano carriers selectively deliver melittin to multiple tumor targets (i.e., endothelial cells and cancer cells) through a novel hemifusion mechanism without disrupting the cell membrane barrier to trigger apoptosis and cause regression of precancerous dysplastic lesions. The ability to restrain the wide-spectrum lytic potential of a potent cytolytic peptide in a nano vehicle combined with the flexibility of passive or active molecular targeting to treat cancer at multiple stages represents an innovative molecular design for chemotherapy with broad spectrum cytolytic peptides.

292 Musculoskeletal Deficits and Muscle Mitochondrial Dysfunction in Adult Long-Term Cancer Survivors

Karen L Syrjala[1,2], Kevin E Conley[3], Sharon A Jubrias[3], Samantha B Artherholt[1,2], Janet R Abrams[1]

[1]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA; [2]Psychiatry and Behavioral Sciences, and [3]Radiology, University of Washington, Seattle, WA

Nearly 12 million cancer survivors diagnosed with an invasive disease are living in the United States with 2 million being added annually by 2020. Chief among the long-term consequences of successful treatment of cancer is an enduring fatigue or lack of stamina, along with muscle weakness and myalgias in up to 70% of survivors. Despite these widely reported symptoms, few if any investigations have examined biomarkers or functional mechanisms for these symptoms. We are establishing models for imaging and other biomarker assessments for musculoskeletal symptoms by first developing measures of subjective symptoms and then applying in vivo functional mitochondrial imaging approaches to examine skeletal muscle energetics and underlying mechanisms using magnetic resonance spectroscopy (MRS) and optical spectroscopy (OS), along with traditional physiologic measures of musculoskeletal function. The method permits quantitative measurement of muscle mass, aerobic capacity, and the function of key mass and energy fluxes (ATP and O2) governed by mitochondria. In initial work with 5–20-year survivors of hematologic malignancy, we have tested body composition with dual energy X-ray absorptiometry scans, aerobic capacity with treadmill testing, grip strength with the hand dynamometer, and self-report of musculoskeletal symptoms prior to MRS and OS testing. Although only 16% were obese by body mass index, 62% were obese by body fat percent, supporting a sarcopenia hypothesis for cancer survivors. We found that 43% were in the poor or very poor range in aerobic capacity. In these survivors, compared with adult norms, we documented mitochondrial energy uncoupling (ATP/O2) accompanied by increased mitochondrial content as indicated by oxidative capacity in both hand and leg muscles. The impairments in mitochondrial function are similar to those seen in neurodegenerative diseases despite the fact that participants had been free of cancer and other major chronic illness for 5 or more years. The findings suggest that mitochondrial changes seen in cancer survivors are due to excess reactive oxygen species and may respond to antioxidant treatment or exercise as has been found to reverse fatigue in some cancer survivors. These methods will support exercise clinical trials to reverse mitochondrial damage in cancer survivors and can be used with animal models to test antioxidant treatments to refine understanding of biologic pathways for long-term musculoskeletal deficits in cancer survivors.

293 Stroma and Stroma-Tumor Interactions as Therapeutic Targets in Breast Cancer

M. Andreeff, X. Ling, Y.-X. Shi, E. Spaeth, K. Watson, V. L. Battula, F. Lang, G. Hortobagyi, F. Marini

The University of Texas M. D. Anderson Cancer Center, Houston

Stromal cells provide structural support for malignant cells, modulate the tumor microenvironment, and influence phenotypic behavior as well as aggressiveness of the tumor. In response, the tumor provides growth factors, cytokines, and cellular signals that recruit new cells into the microenvironment. The tumor microenvironment appears to exhibit cytokine profiles and cellular signals similar to those characteristics of wounded or damaged tissues. We therefore hypothesized that mesenchymal stem cells (MSC) home to and selectively proliferate in the tumor microenvironment and that gene-modified MSC can be used as cellular vehicles to deliver gene products into tumors. Our group indeed demonstrated first that bone marrow-derived MSC integrate into solid tumors (breast cancer, melanomas, gliomas) as stromal elements (Cancer Res 2002, 2006, JNCI 2004) and that MSC develop into tumor-associated fibroblasts (TAF's) (PLoS ONE, 2009). MSC home to and participate in tumor stroma formation in both primary and metastatic tumors, including MDA231 xenografts and 4T1 syngeneic breast cancers. Once homed to tumor beds, MSC proliferate. We then demonstrated that IFN-ß, introduced by adenoviral gene transfer or by electroporation, is produced intra-tumorally and exerts significant antitumor effects, as evidenced by firefly luciferase imaging and extension of survival. We are initiating a clinical trial to test this concept in patients. Alternatively, we investigated the ability to disrupt MSC/tumor interactions by blocking the SDF-1 receptor CXCR4 with peptides and small molecule inhibitors (Plerixafor, Genzyme). Systemic administration of this CXCR4 inhibitor inhibited tumor growth and completely abrogated or significantly (>95%) reduced pulmonary and hepatic metastases in syngeneic 4T1 breast carcinomas and extended survival of the animals. Soluble CD44, produced by gene-modified MSC, prevented homing of these MSC in the same 4T1 model. Results suggest that bone marrow derived MSC home to tumors and their metastases and participate in stroma formation. MSC can be modified for intra-tumoral production of anti-tumor agents, effectively inhibit tumor growth and metastases, and prolong survival. Alternatively, inhibition of MSC homing by soluble CD44 or disruption of tumor-stroma interactions by CXCR4 inhibition also exerts major anti-tumor activities. Hence, the tumor stroma is a novel target in cancer therapy.

294 Complete Tumor Responses in Lymphoma Patients Who Receive Autologous Cytotoxic T Lymphocytes Targeting Ebv Latent Membrane Proteins

C. M. Bollard[1], S. Gottschalk[1], A. M. Leen[1], A. Sheehan[1], C. G. Carrum[1], H. Liu[1], B Pro[2], L. Fayad[2], A. Younes[2], A. P. Gee[1], M. K. Brenner[1], C. M. Rooney[1], H. E. Heslop[1]

[1]Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children's Hospital and The Methodist Hospital, Houston, TX; [2]The University of Texas M. D. Anderson Cancer Center

EBV-associated Hodgkins Lymphoma (HL) and some non-Hodgkins lymphoma (NHL) have type II viral latency expressing the subdominant EBV antigens LMP1 and LMP2, which may serve as targets for immunotherapy approaches. We hypothesized that CTL enriched for effector cells specifically targeting LMP antigens would have efficacy in patients with EBV+ lymphoma. LMP-CTL were generated using dendritic cells for initial stimulations then EBV-transformed lymphoblastoid cell lines (LCL), both of which had been genetically modified to overexpress either LMP2 alone or inactive LMP1 (dLMP1) and LMP2 by transduction with an Ad5f35LMP2 (n=16) or Ad5f35dLMP1-I-LMP2 (n=14) vector respectively. All LMP-CTL lines were polyclonal comprising CD4+ (mean 17±18%; range 1–92%) and CD8+ (mean 74 ± 25%; range 1–99%) T-cells. Flow cytometric analysis of memory markers revealed mixed populations of CD45RA-CD62L- T-cells (45±15%; range 31–63%) and CD45RA- CD62L+ T-cells (34±5%; range 28–41%). CTL lines had specificity for CD4+ and CD8+ restricted LMP2 epitopes alone (mean 1; range 0–7) or both LMP1 and LMP2 epitopes (mean 2; range 0–6) per CTL line, as determined using overlapping LMP1 and LMP2 peptide pools in ELISPOT assays. Twenty-eight patients with EBV+ HL and NHL have been treated on dose escalation studies, 16 with LMP2 CTLs, and 12 with LMP1/2 CTLs. No immediate toxicity was observed. After CTL infusion, increased numbers of LMP-specific T-cells were detected in the blood of 15/22 evaluable patients, (range 2 to 70 fold) persisting for up to 3 months. Additionally, two patients had lymph node biopsies 3–6 months post CTL, which showed selective accumulation of LMP2-multimer positive T-cells in lymph nodes. Some 14/15 high-risk and/or multiply relapsed patients who received LMP-CTL as adjuvant treatment remain in remission for a median of 2 years (range >3 months – >5 years) after CTL. Some 13 patients had detectable disease at the time of CTL, 2 of these had progressive disease by 8 weeks, and 11 had clinical responses. The median duration of the clinical responses is 1.5 years with 3 partial responses (>3 to 36 months) and 8 complete responses (range >6 months to >4.5 years). In conclusion, immunotherapy with CTL targeting LMP antigens is well tolerated in patients with EBV+ lymphoma, and infused LMP-CTL can accumulate at tumor sites and induce complete and sustained clinical responses in 80% of patients. Our future directions include the administration of LMP-specific T cells rendered resistant to the immune suppressive effects of TGFß secreted by the lymphoma tumor cells, and this study is now actively enrolling patients.

295 Successful Haploidentical Hematopoietic Cell Engraftment Using a Non-Myeloablative Preparative Regimen Including Natural Killer (NK) Cells to Treat Refractory Acute Myeloid Leukemia

Sarah Cooley[1], David McKenna[2], Michael Verneris[3], Bruce R Blazar[3], Philip B McGlave[1], Daniel J. Weisdorf[1], Jeffrey S Miller[1]

[1]Hematology, Oncology and Transplantation, University of Minnesota Masonic Cancer Center, [2]Clinical Cell Therapy Laboratory, Molecular & Cellular Therapeutics, and [3]Pediatrics, University of Minnesota

We have previously shown that adoptive transfer of haploidentical natural killer (NK) cells can induce remissions in 27% of patients with refractory acute myeloid leukemia (AML). Because those remissions were not durable, we added a CD34+ stem cell infusion to create a potentially curative non-myeloablative haploidentical transplantation protocol. The lymphodepleting chemoradiation plus NK cell preparative regimen includes fludarabine 25 mg/m2 x 5, cyclophosphamide 60 mg/kg x 2, and 200 cGy of total body irradiation (added to further improve NK cell expansion). The NK cell product, prepared by cliniMACS (Miltenyi) CD3-depletion of a single leukapheresis collection from a haploidentical donor, is incubated overnight in 1000 U/ml IL-2 prior to infusion and followed by 6 doses subcutaneous IL-2 (10 million units) given every other day to promote in vivo NK cell expansion. A CD34-selected filgrastim-mobilized peripheral blood graft from the same donor is given with 3 doses of Thymoglobulin 3 mg/kg as the only additional immunosuppression. In the first cohort of 13 patients, a significantly higher rate of NK cell expansion (75% [9/12 evaluable]; mean 607±184 NK cells/ml) was achieved compared to the adoptive NK cell transfer regimen, which did not include radiation. Plasma IL-15, which is critical for NK expansion, was highest after the preparative regimen prior to the NK infusion (64 ± 8 pg/ml [day –12] vs. 6 ± 1 pg/ml [baseline pre-chemo]; $p < .0001$). This adoptive NK cell plus allograft protocol led to 66% of relapsed or refractory AML patients (8/12 evaluable) clearing leukemia by day –1, with only one late relapse (day +93). Patients who did not clear leukemia (N=4) did not engraft, and it was not evaluable in three patients with early treatment related mortality (TRM). All others (N=6), engrafted quickly (defined by an absolute neutrophil count >500/ml and 100% donor chimerism, median 17 days). None developed graft vs. host disease (GVHD), but infections were common. One patient is alive in remission beyond 1 year. This work highlights the impact of the translational research facilities at the University of Minnesota, which allow for the pre-clinical development, validation, and clinical testing of novel cellular immunotherapies. We have demonstrated that addition of haploidentical NK cells to a non-myeloablative haploidentical transplantation yields NK cell expansion and achievement of complete remissions in patients with refractory AML. We continue to build upon this promising platform by adding other strategies aimed at improving disease-free survival in patients with refractory AML.

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296 Development of Therapeutic Approaches to Increase Potency of Graft-versus-Tumor Responses in Patients With Persistent Hematologic Malignancies After Allogeneic Hematopoietic Stem Cell Transplantation

Nancy Hardy, Frances Hakim, Michael Bishop

Experimental Transplantation and Immunology Branch (ETIB), Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD

Allogeneic hematopoietic stem cell transplantation (allotransplant) is a potentially curative option for patients with refractory hematologic malignancies. Its efficacy is immune-mediated and referred to as the graft-versus-tumor (GVT) effect. Not all patients are cured with allotransplant, however, and the biologic mechanisms for its success or failure are not understood. The management of persistent tumor is a significant clinical problem following allotransplant. Standard approaches include withdrawal of immune suppression and/or administration of additional donor lymphocytes (DLI). While successful in some patients with early relapse for indolent tumors, when it does not control tumor there are no standard, curative treatment options. ETIB has a major programmatic initiative to study clinical and biologic features of patients with persistent tumor after allotransplant and evaluate treatment strategies aimed at enhancing an inadequate GVT response. We have learned that the timing of disease progression relative to donor T cell engraftment influences patient outcomes. Preliminary data suggest that tumor-infiltrating lymphocytes are found after allotransplant and that they are of donor origin. We have observed that costimulation ex-vivo generates large numbers of donor T cells with an effector phenotype. One hypothesis we are testing is that tumor-specific lymphocytes are a component of these tumor-infiltrating donor lymphocytes, and costimulation ex-vivo will generate a donor effector-T cell product with increased tumor efficacy relative to standard DLI. We are currently performing a study on the feasibility, safety, and efficacy of administering tumor-derived, costimulated donor lymphocytes to patients with persistent hematologic malignancy after allotransplant. Additional studies are testing other means of enhancing donor immune responses, (e.g., whether radiation can improve GVT responses, hypothesizing that radiation-induced tumor damage may enhance antigen presentation and tumor-reactive donor T cell activation in vivo). We are collaborating with other NCI investigators to identify and test strategies that may work synergistically with GVT (e.g., treating with an anti-CTLA4 monoclonal antibody, hypothesizing that blocking suppressor T cells may unleash GVT responses; and administration of genetically modified donor T cells that express anti-CD19 chimeric antigen receptors as a means of targeting donor responses toward B-cell tumor-associated antigens).

297 Genetic Manipulation and Propagation of Clinical Grade CD19-Specific T Cells Derived from Umbilical Cord Blood

Partow Kebriaei[1], Susan S. Kelly[2], Harjeet Singh[2], Margaret J. Dawson[2], Matthew J. Figliola[2], Helen Huls[2], Elizabeth J. Shpall[1], Richard E. Champlin[1], Laurence J. N. Cooper[2]

Divisions of [1]Stem Cell Transplantation and Cellular Therapy and [2]Pediatrics, University of Texas M. D. Anderson Cancer Center, Children's Cancer Hospital, Houston TX

Allogeneic umbilical cord blood (UCB) has been widely used as a source of allogeneic hematopoietic stem cells (HSC) to treat a range of disorders. The rapid procurement, lower risk of graft-versus-host-disease (GvHD) and ability to transplant across HLA disparities make it an attractive stem-cell source. However, relapse of malignancy and GvHD remain significant problems. Adoptive transfer of tumor-specific T cells from bone marrow or peripheral blood has been a promising approach to prevent and treat relapse after allogeneic HSC transplantation (HSCT). Although similar benefit is anticipated after UCB transplantation (UCBT), in practice this approach is constrained by the low number of hematopoietic cells in the UCB unit, the inability to recollect the donor, and difficulty in generating antigen-specific T cells from functionally naive T cells. Genetic introduction of chimeric antigen receptors (CARs) can redirect the specificity of T cells for desired tumor antigens. To target B-lineage malignancies, we have constructed CARs to redirect the specificity of T cells for CD19, a B-lineage-specific cell-surface molecule, independent of major histocompatibility complex (MHC). To generate CD19-specific T cells we have used the Sleeping Beauty (SB) system to improve non-viral gene transfer efficiency and have recently received regulatory approval from the NIH-OBA for first-in-human application of autologous CAR+ T cells after HSCT. To numerically expand genetically modified T cells from limiting amounts of hematopoietic starting material, we have selectively propagated CAR+ UCB-derived T cells on irradiated CD19+ artificial antigen presenting cells (aAPC), generated in collaboration with Dr. Carl June. These aAPC were derived from K562 and modified to co-express the desired the co-stimulatory molecules CD137L, CD86, and membrane-bound IL-15. These aAPC have been manufactured as a clinical grade master cell bank (MCB) by PACT under the auspices of the NHLBI. The electroporated and propagated cells are CD8+ and CD4+ memory and effector T cells that exhibit redirected CD19-dependent killing and cytokine production. The adoptive transfer of donor-derived CD19-specific T cells after UCBT will be tested as an approach to enhance the graft-versus-tumor-effect without increasing GvHD. The relative ease and low cost associated with production of clinical grade DNA plasmids, electroporation, and reproducible outgrowth of T cells stably expressing CAR on thawed γ-irradiated MCB of aAPC should enable adoptive immunotherapy trials using CAR+ T cells.

298 Human Dendritic Cells Adenovirally-Engineered to Express Three Defined Melanoma Tumor Antigens Activate Polyclonal T Cells: Preclinical Development for a Phase II Trial

Jennifer Landsberg[1], Jian Shi[1], Lazar Vujanovic[1], Michelle Messmer[1], John M. Kirkwood[2], **Lisa H. Butterfield**[1]

[1]Department of Medicine, [2]University of Pittsburgh Cancer Institute, and [3]Department of Surgery University of Pittsburgh, Pittsburgh, PA

DC-based immunotherapy has shown promising but infrequent evidence of clinical activity. Many trials have tested immunotherapy with single epitopes and single antigens to activate a single specificity of T cell, often CD8+ only. We previously found that determinant spreading and overall breadth of antitumor immunity correlates with superior clinical response. To promote activation and expansion of polyclonal, multiple antigen-specific CD8+ T cells, as well as to provide cognate help from antigen-specific CD4+ T cells, we have created an adenovirus encoding three full-length melanoma tumor antigens (tyrosinase, MART-1 and MAGE-A6). We previously showed that adenovirus-mediated antigen engineering of human DC is superior to peptide pulsing and has positive biological effects on the DC, allowing efficient activation of not only antigen-specific CD8+ and CD4+ T cells but also NK cells. Here we describe cloning of two different genetic structures of "AdVTMM," an E1/E3-deleted AdV encoding three melanoma antigens. The virus expressed mRNA and protein for all three antigens and AdVTMM-transduced DC activate T cells that recognize melanoma tumor cells more efficiently than single-antigen AdV. Use of these multi-antigen engineered DC may provide for superior immunity and ultimately, improved antitumor responses. We will combine this AdVTMM/DC vaccine with high dose IFN assigned at random, to determine the possible enhancing effects and improved determinant spreading with this combination.

299 Genetically Enhanced Dendritic Cell Immunotherapy for Cancer

Natalia Lapteva, Priya Narayanan, Mamatha Seethammagari, Jan Ole Kemnade, **David M. Spencer**

Baylor College of Medicine, Houston TX

Given the pre-eminent role of dendritic cells (DCs) as antigen-presenting cells, their exploitation as natural adjuvants in vaccination protocols for the treatment of various malignancies and infectious diseases is not surprising. Nevertheless, inadequate or unsustained activation has likely limited response rates in clinical trials. We have previously reported a potent synthetic dimerizer drug-inducible CD40 (iCD40) receptor that permits temporally controlled DC-specific activation within the context of an immunological synapse (Hanks et al, Nat Med, 2005). We showed that iCD40-modified DCs (iCD40-DCs) have enhanced survival, co-stimulatory marker expression, migration, IL-12 production, antigen (Ag)-specific T cell stimulatory capacity, and anti-tumor responses. A phase I/II clinical trial based on iCD40-DCs to treat castration-resistant, metastatic prostate cancer began enrolling patients in July 2009 and will be described. To further improve and simplify this approach that still requires additional TLR stimulation ex vivo for maximum efficacy, we have developed a unified, inducible DC switch, called "iCD40.MyD88," which combines signaling elements from CD40 and TLR adapter, MyD88. Both murine and human iCD40.MyD88-DCs secrete high ng levels of IL-12 and maturation markers in a ligand-dependent fashion. Moreover, tumor-bearing mice treated with antigen-pulsed, iCD40.MyD88-DCs show a potent anti-tumor immune response even more potent than iCD40/LPS-DC-treated animals. This "combo-switch" should permit potent, targeted activation of antigen-pulsed DCs in vivo, leading to improved anti-tumor immunity with reduced side effects. Anti-tumor efficacy and our efforts to convert these reagents to "off-the-shelf" therapies will also be discussed.

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300 Adoptive Cellular Therapy for Glioblastoma Using RNA-Modified T Cells and Dendritic Cells

Duane A. Mitchell[1,2], Isaac Karikari[2], Shireen Khoury[2], Xiuyu Cui[1,2], Weihua Xie[1,2], Robert Schmittling[1,2], Gary Archer[1,2], Darell D. Bigner[3], John H. Sampson[1,2,3]

[1]Duke Brain Tumor Immunotherapy Program, Departments of [2]Surgery (Neurosurgery and Neuro-Oncology), and [3]Pathology, Preston Robert Tisch Brain Tumor Center at Duke, Duke University Medical Center, Durham, NC

Background: Conventional therapies for GBM fail to target tumor cells exclusively. Adoptive cellular therapy targeting tumor-specific antigens holds the promise of eliminating invasive tumor cells with exquisite precision and minimal toxicity. The discovery (Cobbs. et al. 2002) and recent confirmation (Mitchell et al. 2008) that GBM, but not surrounding normal brain tissue, serves as a refuge for Cytomegalovirus (CMV) reactivation provides an unparalleled opportunity to subvert, as tumor-specific antigens, the highly-immunogenic viral proteins expressed by CMV. In this study, we explored the capacity to enhance the immunologic function of CMV-specific T cells and antigen-loaded dendritic cells (DCs) using RNA-based gene modification for use in adoptive cellular therapy protocols.

Patients and Methods: DCs and CMV-specific lymphocytes expanded from the peripheral blood of patients with newly-diagnosed GBM were used to evaluate the efficiency of RNA-based gene modification and capacity to augment immunologic function in vitro and in vivo.

Results: We observed that the efficient transfection of T cells using RNA electroporation requires prior activation of T cells using mitogens or antigen-specific stimulation. This observation was leveraged to allow for the selective enrichment and modification of CMV-specific T cells. Furthermore, we demonstrated that CMVs-specific T cells can be functionally modified using RNA transfection of the C-X-C chemokine receptor, CXCR2, to migrate efficiently toward a variety of chemokines secreted by gliomas in vitro and in vivo. These studies demonstrate the utility of RNA transfection as a simple method to purify and selectively modify the function of antigen-specific T cells for use in adoptive immunotherapy and importantly provide evidence that transient expression of proteins using RNA transfection is an efficient means of modulating the in vivo function of activated T cells. Additional studies examining the modification of antigen-pulsed DCs in vitro are underway in order to enhance the effector function of CMV-specific T cells prior to adoptive transfer.

Conclusions: RNA-based gene modification of T cells and DCs is an effective means to enhance the immunologic function of cells for use in adoptive cellular therapy.

301 Novel Immune Based Preparative Regimen for Allogeneic HCT

Robert S. Negrin, Robert Lowsky, Samuel Strober

Stanford University, Stanford, CA

Immune regulation is critical in health and disease. Following allogeneic HCT is one of the clearest examples of the role of effective immune responses to eradicate residual disease termed the graft vs tumor (GVT) effect, whereas a dysfunctional immune response can result in graft vs host disease (GVHD), which is the major limitation of treatment. Preclinical animal studies have identified immune regulatory pathways as key components to reducing the risk of GVHD while maintaining GVT. Two strategies have been developed exploiting the potential biological activity of immune regulatory pathways that show promise in the clinic. The first involves the use of total lymphoid irradiation (TLI) and anti-thymocyte globulin (ATG), which resulted in reduced GVHD risk even when up to 1,000 times the number of T cells were infused in preclinical studies. The mechanism underlying the reduction in GVHD risk is due to the alteration of the ratio of conventional T cells to regulatory natural killer - T (NK-T) cells. NK-T cells are capable of suppressing aberrant immune responses through the recruitment of other regulatory T cell populations and through the release of certain cytokines. Translation of this protocol to the clinic has been achieved where the risk of GVHD and 1-year transplant related mortality is reduced to approximately 5% following allogeneic HCT in over 150 patients from related and unrelated donors. This strategy is currently being evaluated in pivotal trials for the treatment of patients with malignancies as well as for the induction of tolerance. A second approach using highly purified regulatory T cells has also been studied extensively in animals models and is in the early phases of clinical translation. Regulatory T cells have been shown to suppress the early proliferation of alloreactive T cells, thereby suppressing the GVHD response, whereas activation of cytotoxic T cells still occurs maintaining GVT responses. These approaches highlight clinical translation of novel concepts in cellular immunology to suppress certain immune reactions while maintaining others. These concepts have potential broad applicability for the treatment of a variety of diseases such as cancer and autoimmune disorders and for the induction of tolerance to transplanted solid organs.

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302 Development of a Novel Human Xenograft Model for the Study of Skin Cancer Biology In Vivo [WITHDRAWN]

Yosef Refaeli[1], Antonio Jimeno[2], Xiao-Jing Wang[3], Dennis Roop[1], Marianne Berwick[4], David Norris[1]

Departments of [1]Dermatology, [2]Medicine, and [3]Pathology, University of Colorado Denver, Aurora, CO; [4]Division of Epidemiology and Cancer Therapy & Research Center, University of New Mexico, Albuquerque

Although the direct patient tumor xenograft model (DPTXM) is a powerful tool, host animals are immunocompromised, limiting the utility of this system for studying interactions between tumors and the immune system. Humanized chimaeric mice transplanted with human fetal liver HSCs or cord blood HSCs have been developed to address this issue. However, at least 106 HSCs are required for engraftment, and reconstitution is still a rare event. Also, since primary HSCs need to be freshly implanted and additional HSCs cannot usually be obtained from the same source, the results from one cohort of mice are restricted to that experiment. Finally, because transplanted human lymphoid cells remain restricted to the thymus, bone marrow, and spleen, this system cannot be used to study tumor-immune system interactions. By transplanting human conditionally transformed long-term HSCs (ctlt-HSC) into sublethally irradiated immunodeficient mice, we have developed a novel chimaeric mouse with a human hematopoietic system. Our novel approach provides several advantages over older systems. First, because we use a conditionally immortalized HSC cell line as the source, variability is reduced, facilitating comparisons across experimental conditions. Second, we can begin the process with far fewer cells than current approaches, since we will generate HSC cell lines for xenotransplantation. Third, our humanized chimaeric mice produce mature human T and B cells that can infiltrate into tumors via blood vessels and lymphatic vessels; hence, these animals can be used to study tumor-immune system interactions. The goal of Project 2 is to establish a tumor xenograft model in chimaeric NOD/SCID/ß2M–/– mice reconstituted with human ctlt-HSCs. This system will be used to evaluate CSC properties, niche, and CSC-host interactions and to test therapeutic strategies.

We use two strategies to develop humanized xenochimaeric, tumor-bearing mice. First, we will obtain both tumor and blood samples from an individual patient. We will conditionally immortalize HSCs isolated from peripheral blood, then transplant them into sublethally irradiated NOD/SCID/ß2M–/– mice. Meanwhile, tumors will be passaged by xenografting in nude mice. Once NOD/SCID/ß2M–/– have been reconstituted with ctlt-HSC, we will then transplant tumors derived from the same patient into these animals. In parallel, we will also develop a system in which we use a universal donor ctlt-HSC line and induce T cell tolerance to an allogeneic (non-self) tumor. These unique systems will allow us to study tumor-immune system interactions in vivo.

303 Genetic Engineering of a Cancer-Targeted Human Immune System

Antoni Ribas[1], Thinle Chodon[1], Begonya Comin-Anduix[1], Erika von Euw[1], Richard C. Koya[1], Caius Radu[1], James S. Economou[1], Owen N. Witte[1], David Baltimore[2]

[1]University of California, Los Angeles; [2]California Institute of Technology

Several immune-based strategies result in infrequent but durable remissions of metastatic melanoma. In our 10-year experience in investigator-initiated clinical trials using dendritic cell vaccines and CTLA4-blocking monoclonal antibodies, we have concluded that a rate-limiting step is the number of T cells that can effectively home to the tumor and carry out cytotoxic killing. To circumvent this limitation, we have tested T cell receptor (TCRs) gene transfer to redirect lymphocytes, studied tumor targeting of TCR-redirected lymphocytes using molecular imaging techniques in animal models, generated GMP viral vectors for therapeutic TCRs, and developed PET reporter gene systems and probes to follow immune cell lifespan and localization in vivo. Pre-clinical testing using mouse models of tumor immunity has demonstrated that genetic modification of lymphocytes and stem cells with tumor reactive TCRs and their adoptive transfer to tumor bearing hosts produce sustained immune responses based on prolonged production of T killer cells and memory populations. We have taken this experience to the clinic. Early data from our ongoing clinical testing using a MART-1 reactive TCR modified peripheral T cells demonstrates target specificity, effective homing to tumor sites, and objective tumor responses by clinical and PET imaging criteria in the first two patients on study. We obtained high transduction efficiencies in clinical grade cell preparations, with over 70% of the lymphocytes reinfused to patients being positive for the transgenic TCR detected by tetramer assay. These cells persist in vivo, with frequencies of 40–60% in the first 2 weeks, and maintained at over 30% by 3 months. Regressing melanoma lesions have dense infiltration by CD8+ T lymphocytes, and tetramer analysis of tumor infiltrating lymphocytes (TILs) show at least 10% of the population bearing MART-1 specificity. Our continued translational plans include the generation of clinical grade lentiviral vectors expressing TCRs and PET reporter genes to allow the genetic modification of autologous HSCs to produce an immune system tailored to react against the patient's tumor, which can continually produce T cells over long periods of time to treat bulky tumor and prevent recurrence, and image hematopoietic and immune system reconstitution. In conclusion, our translational program is making progress toward a goal of efficiently genetically engineering the human immune system to target cancer.

304 Cellular Immunotherapy Trials Targeting Human Cytomegalovirus Antigens in Glioblastoma

John H. Sampson[1,2,3], Gary Archer[1,2], Darell D. Bigner[3], Henry S. Friedman[2,3], Denise Lally-Goss[1,2], Beth Perry[1,2], James E. Herndon, II[4], Sharon McGehee[1,2], Roger McLendon[3], David Reardon[2], Duane A. Mitchell[1,2]

[1]Duke Brain Tumor Immunotherapy Program, Departments of [2]Surgery (Neurosurgery and Neuro-Oncology), [3]Pathology, and [4]Biostatistics and Bioinformatics, Preston Robert Tisch Brain Tumor Center at Duke University, Durham, NC

Background: Conventional therapies for GBM fail to target tumor cells exclusively. Immunologic targeting of tumor-specific proteins may allow more precise eradication of neoplastic cells. The discovery (Cobbs. et al. 2002) and recent confirmation (Mitchell et al. 2008) that GBM, but not surrounding normal brain tissue, serves as a refuge for Cytomegalovirus (CMV) reactivation provides an unparalleled opportunity to subvert, as tumor-specific antigens, the highly-immunogenic viral proteins expressed by CMV.

Patients and Methods: A phase I/II randomized, prospective clinical trial was undertaken to assess the immunogenicity and efficacy of targeting the immunodominant CMV integument protein, pp65, in patients with newly-diagnosed GBM using pp65 RNA transfected dendritic cells (DCs). After resection and radiation with concurrent TMZ (75mg/m2/d), patients received subsequent monthly cycles of TMZ (200 mg/m2) simultaneous with intradermal DC vaccinations. Subjects received vaccinations until there was evidence of tumor progression or death.

Results: Thirteen patients were treated with pp65 RNA-pulsed DC vaccines on this protocol. There were no vaccine-related, reportable serious adverse events. One nearly complete response was observed. Median progression-free survival (PFS) is 15.4 months (CI95: 10.0, ∞) and median overall survival (OS) is 20.6 months. Increases in CMV-specific delayed-type hypersensitivity (DTH) reactions, humoral responses, and circulating CMV-specific T cells were observed after vaccination; however, polyfunctional immune responses remained impaired. Ex vivo expansion of CMV-specific T cells, however, restored polyfunctional immune responses in these patients. Therefore, a phase I clinical trial of adoptive cellular therapy targeting CMV antigens in patients with newly-diagnosed GBM has been initiated at our Center.

Conclusions: Immunotherapy targeting CMV pp65 is safe in patients with GBM and produces promising immunologic, radiographic, and clinical responses. Adoptive T cell therapy may improve induction of polyfunctional immune responses in these patients and is under current evaluation.

305 Adoptive Immunotherapy With Cytotoxic T Lymphocytes Grafted With Specific Chimeric Antigen Receptors for Therapy of Patients With NHL or HL

Barbara Savoldo, Gianpietro Dotti, Catherine Bollard, Carlos Ramos, Adrian Gee, Cliona Rooney, Malcolm Brenner, Helen Heslop

Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children's Hospital, The Methodist Hospital

Adoptive immunotherapy with antigen-specific cytotoxic T lymphocytes (CTLs) is clinically effective for Epstein-Barr virus (EBV)-associated malignancies and melanoma. More recently, strategies have been developed to redirect the specificity of polyclonal T lymphocytes by gene transfer of chimeric antigen receptors (CARs) that combine a single chain antibody with the zeta-chain of the TCR/CD3 complex. This approach may extend the beneficial effects of T cell therapies to several human malignancies. Although effective in vitro and in animal experiments, CAR-modified T lymphocytes initially had only limited success in clinical trials, due in part to the lack of essential co-stimulatory signals to the T cells after CAR engagement with the antigen, leading to rapid disappearance of transgenic cells in vivo. To overcome this limitation, two approaches have been devised: (1) expression of CARs in T lymphocytes with defined antigen specificity, such as EBV-specific CTLs, that will receive the appropriate co-stimulation through their native alpha-betaTCR when they encounter cognate antigens on professional APCs; (2) expression of "2nd generation" CARs that directly incorporate co-stimulatory endodomains (CD28, OX40 or 4-1BB), allowing activation of the T cells and IL-2 production. In a previous phase I clinical trial in neuroblastoma patients, we have validated in vivo the concept that EBV-specific CTLs engrafted with a "1st generation" CAR targeting the neuroblastoma antigen GD2 persist longer than activated polyclonal T lymphocytes expressing the same CAR and remain functional following engagement of either their native or chimeric receptors. We have recently initiated a phase I clinical trial in patients with NHL to test whether polyclonal T cells grafted with a "2nd generation" CAR targeting the CD19 molecule and incorporating the CD28 endodomain have enhanced survival as compared to T cells expressing the same CAR lacking the CD28 endodomain. In the first patient enrolled, T cells expressing the "2nd generation" CAR persisted longer than those engrafted with the "1st generation" CAR. In a second clinical trial for patients with NHL, we are comparing EBV-specific CTLs engrafted with the "1st generation" CAR targeting CD19 with polyclonal T cells expressing the "2nd generation" CAR. We are also implementing similar studies in patients with CLL/NHL and in patients with Hodgkin Lymphoma to test safety and efficacy of CAR-redirected T cells targeting the kappa-light chain of the immunoglobulin and the CD30 molecule, respectively.

306 An Optimized Adenovirus Vaccine for Boosting the Expansion and Antitumor Activity of Adoptively Transferred Antigen-Specific T Cells

Xiao-Tong Song[1], Meghan Turnis[1], Bang-Xing Hong[2], Lisa Rollins[1], Si-Yi Chen[2], Helen E. Heslop[1], Cliona M. Rooney[1], **Stephen Gottschalk**[1]

[1]Baylor College of Medicine, Houston, TX; [2]University of Southern California, Los Angeles

Background: We have shown that the infusion of Epstein-Barr virus (EBV)-specific T cells results in complete remissions in patients with EBV-associated malignancies who have low disease burden. However, infused EBV-specific T cells did not expand significantly in vivo, limiting their antitumor activity. While lymphodepletion with cytotoxic agents is one strategy to enhance the expansion of adoptively transferred T cells, its use is limited by unwanted side effects. The aim of this project was to develop an adenoviral vaccine to boost the expansion of adoptively transferred T cells without systemic side effects. We show here that a vaccine that (1) provides antigens, (2) inhibits the antigen presenting attenuator A20, and (3) encodes flagellin for Toll-like receptor (TLR) 5 activation boosts the expansion and antitumor effects of adoptively transferred T cells in the B16-OVA tumor model.

Methods: Recombinant adenoviruses were constructed that express A20-shRNA to silence A20 (Ad-shA20) or A20-shRNA and flagellin (Ad-shA20-FL). B16-OVA tumor-bearing mice were either vaccinated with (1) Ad-shA20-FL/Ad-OVA, (2) Ad-shA20/Ad-OVA, (3) Ad-OVA or (4) Ad-shA20-FL before the adoptive of OVA-specific T cells (OT-I T cells). Routine immunological assays were used to determine the effects of vaccine/T-cell therapy. Bioluminescence imaging was used to track infused OT-I T cells, and knockout mice were used for mechanistic studies.

Results: B16-OVA tumor-bearing mice were vaccinated on day 5 with Ad-shA20-FL/Ad-OVA, Ad-shA20/Ad-OVA, or Ad-OVA and received a single dose of OT-I T cells on day 7. Only the Ad-shA20-FL/Ad-OVA vaccine induced significant expansion of OT-I T-cells resulting in prolonged regression of B16-OVA tumors. In contrast, injection of OT-I T cells or the Ad-shA20-FL/Ad-OVA vaccine had only modest antitumor effects. Mechanistic studies revealed that Ad-shA20-FL/Ad-OVA vaccination induced robust Th1, Th2, and Th17 responses in B16-OVA bearing mice. Activation of Th1 T cells was critical for the observed effects since the Ad-shA20-FL/Ad-OVA vaccine was ineffective in activating and expanding infused OT-I T-cells in IL12–/– (Th1 deficient) but not in RORγt–/– (Th17 deficient) mice.

Conclusion: We show here that a vaccine that induces a strong Th1-polarized environment boosts the expansion and antitumor effects of adoptively transferred T cells. The developed vaccine is a promising candidate to improve current T-cell therapy approaches for malignancies.

307 Selective Retargeting of Adenoviruses to Metastatic Breast Cancer

Shilpa Bhatia[1], Ana Nedeljkovic-Kurepa[1], Yoshinobu Odaka[1], Angel A. Rivera[2], David T. Curiel[2], Alexander Pereboev[2], and **J. Michael Mathis**[1]

[1]Gene Therapy Program, Department of Cellular Biology and Anatomy, Louisiana State University Health Sciences Center, Shreveport, LA; [2]Division of Human Gene Therapy, Departments of Medicine, Surgery, and Pathology, University of Alabama at Birmingham

The success of gene therapy relies on efficient and targeted delivery systems. The concept of retargeting vectors is particularly important in view of limited success of currently available adenovirus (Ad) gene therapy approaches for metastatic disease. One major hurdle for systemically administered Ads is promiscuous tropism that leads to infection of non-target cells, notably liver. This can be circumvented by modifying Ad native tropism and redirecting to different receptors expressed on target cells. Many solid tumors and hematopoietic malignancies overexpress the chemokine receptor CXCR4, a G protein-coupled receptor that plays a major role in cancer metastasis. In this study, we explored retargeting Ads to the CXCR4 receptor overexpressed on breast cancer cells. We designed and tested a recombinant bispecific adapter protein (sCAR-Linker-CXCL12) that fuses the extracellular domain of native adenovirus receptor (sCAR) to human CXCL12 chemokine, the ligand for the CXCR4 receptor.

To optimize the virus to adapter protein ratio, infectivity assays were performed in the absence and presence of adapter in a panel of human breast cancer cell lines and normal cells. Cells were infected with different titers of Ad5-CMV-GFP-Luc with and without sCAR-Linker-CXCL12. Forty-eight hours post-infection, cells were harvested and analysed for reporter gene expression by fluorescence microscopy and flow cytometry. Biological activity of the adapter molecule was also determined by incubating the cells with different concentrations of sCAR-Linker-CXCL12 protein in the presence of Ad5-CMV-GFP-Luc. In addition, the binding specificity of the adapter protein to the Ad5 fiber and CXCR4 receptor was analyzed by indirect ELISA. Importantly, ELISA assay demonstrated strong binding of the bispecific adapter molecule to both Ad5 fiber and CXCR4 receptor. A marked enhancement in the infectivity of breast cancer cells was observed using the sCAR-Linker-CXCL12 retargeted Ad compared to the untargeted vector. In contrast, a protective effect was evident in the normal cells that could be attributed to the adapter molecule.

In this study, we report that sCAR-Linker-CXCL12 can dramatically redirect an Ad gene therapy vector to CXCR4-positive breast cancer cells. This bispecific adapter protein could be a powerful agent to retarget Ads to tumor metastases. A future goal is to investigate the capacity of this agent to redirect Ads in vivo using breast cancer metastasis models.

308 Development of a Novel Antiandrogen for Castration-Resistant Prostate Cancer

Nicola Clegg[1], John Wongvipat[1,2], Chris Tran[1], Anna Dilhas[3], Hong Zhao[3], Guangbin Yang[3], Teresa Wasielewska[1], Samedy Ouk[9], Peter Smith-Jones [4], Michael Jung (9), Mark Klang[5], Elisa De Stanchina[6], Nian Wu[7], Ouathek Ouerfelli[3], Howard I. Scher[8], Charles Sawyers[1,2]

[1]Human Oncology & Pathogenesis Program, [2]Howard Hughes Medical Institute, [3]Organic Synthesis Core Facility, [4]Molecular Pharmacology & Chemistry, [5]Research Pharmacy Core Facility, [6]Antitumor Assessment Core Facility, [7]Analytical Pharmacology Core Facility, [8]Genitourinary Oncology Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY; [9]Department of Chemistry, University of California, Los Angeles, CA

Nearly 30,000 men in the United States die each year from castration-resistant prostate cancer (CRPC). Recent work has established that restored androgen receptor (AR) function is a common feature of CRPC. Studies in human tumors and xenograft models have implicated several mechanisms of AR reactivation in the setting of androgen ablation, including AR gene amplification, AR gene mutation, increased AR mRNA and/or protein expression (without known genetic alteration), and increased biosynthesis of androgens in prostate tumors. The clinical relevance of restored AR function in disease progression has been recently established in phase 1/2 clinical trials in men with CRPC, based on the activity of two novel agents, abiraterone acetate and MDV3100. MDV3100 is a competitive AR antagonist discovered by our group in a screen for compounds with improved AR binding affinity and more potent antagonism of androgen activity, relative to bicalutamide. Unlike bicalutamide, MDV3100 blocks binding of AR to DNA and impairs AR nuclear translocation, indicative of a novel mechanism of AR inhibition. One hundred and forty men with CRPC have been treated with MDV3100 in a phase I/II study that, in 56% of cases, revealed a 50% or greater decline from baseline in PSA levels (sustained for at least 12 weeks), together with radiographic evidence of stable disease or regression. Meanwhile, a continued search for improved antiandrogens has identified additional compounds, called the A-series, with even more potent antiandrogen activity in comparison to MDV3100 and superiority in several preclinical assays. Using the developmental therapeutics infrastructure available at MSKCC, we selected A52 as the clinical candidate. We have completed scale-up of the chemical synthesis and are in the midst of toxicology studies in rodents and dogs, as required for submission of an IND application to the FDA.

309 Phase II Trial of Exemestane in Postmenopausal Women at Risk for Breast Cancer: Study Update

Jennifer Eng-Wong[1], Robert Warren[1], Philip Cohen[1], Claudine Isaacs[1], David Venzon[2], Suparna Wedam[3], Jo Anne Zujewski[3], Larissa Korde[4]

[1]Lombardi Cancer Center, Georgetown University; [2]Biostatistics and Data Management Section, Center for Cancer Research, [3]Medical Oncology Branch, and [4]Division of Cancer Epidemiology and Genetics, National Cancer Institute

Background: The aromatase inhibitors (AIs) show promise for breast cancer prevention in postmenopausal women. We are conducting a phase II trial of exemestane in women at increased risk for breast cancer. The primary endpoint is change in mammographic density. Secondary endpoints include change in bone mineral density, steroid sex hormones, cholesterol, and breast tissue expression of proliferating cell nuclear antigen and trefoil protein 1, and adverse events. The trial is currently accruing, and we present our experience to date.

Methods: Subjects are screened and enrolled at two sites: Center for Cancer Research, National Cancer Institute (NCI), and Lombardi Cancer Center, Georgetown University Hospital (GUH). Eligible participants are at increased risk for breast cancer by virtue of the following: Gail model risk > 1.7% over 5 years; high risk pathological lesion (e.g., lobular neoplasia, ductal carcinoma in situ); known BRCA1/2 deleterious mutation or prior stage I/II breast cancer at least 2 years from breast cancer treatment and not treated with AIs. Women are required to undergo DEXA scan of the AP spine and hip at baseline and were excluded if AP spine T-score was ≤–2.5 (osteoporosis). Study participants receive exemestane 25 mg, calcium carbonate 1200 mg, and vitamin D 400 IU daily for 2 years. Subjects are evaluated after starting study drug at 1, 3, 6, 12, 18, and 24 months.

Results: As of July 1, 2009, 41 women have enrolled in the trial: 25 at NCI over 48 months and 16 at Georgetown over 13 months. Three subjects at GT were screen failures; two due to osteoporosis, one for study logistic issues. Of the 41 subjects, 3 were eligible by prior breast cancers, 19 by DCIS, 15 by a high risk pathological lesion (atypical hyperplasia or lobular neoplasia), and 4 by Gail Model only; additionally, one subject is a known BRCA mutation carrier. Eleven subjects had taken tamoxifen or raloxifene for risk reduction prior to enrollment. Twenty-five subjects have completed 1 year of exemestane (21 at NCI and 4 at GUH). Four women discontinued agent within the first year (three at NCI and one at GUH.) Of the 25 subjects who completed 1 year of exemestane, all underwent a baseline research breast biopsy and 23 underwent the 12-month biopsy. Grade 3 adverse events possibly, probably, or definitely related to drug included fatigue and headache in one subject; all other adverse events have been grade ½ to date.

Conclusions: We expect to meet accrual target of N=45 in the next 6 months at GUH. Women at risk for breast cancer seek alternatives to selective estrogen receptor modulators and are willing to undergo research breast biopsies.

310 Sulforaphane Destabilizes the Androgen Receptor in Prostate Cancer Cells by Inactivating Histone Deacetylase 6

Angela Gibbs,* Jacob Schwartzman,* Vivianne Deng, **Joshi Alumkal** (*equal contribution)

Division of Hematology & Oncology, Oregon Health & Science University

Background: High consumption of broccoli is associated with reduced prostate cancer risk in epidemiological studies. Sulforaphane, derived from compounds in broccoli, prevents and induces regression of prostate and other malignancies in pre-clinical models, but its mechanisms are not fully defined. Recent reports show that sulforaphane may impair prostate cancer growth through inhibition of histone deacetylases, which are upregulated in cancer. Indeed, one of these enzymes, histone deacetylase 6 (HDAC6), influences the acetylation state of a key androgen receptor (AR) chaperone, HSP90. It is clear that the AR is the central signaling pathway in prostate cancer, and its inhibition is used to both prevent and treat this disease. However, sulforaphane's effects on AR function are unknown. We hypothesized that sulforaphane treatment would lead to hyperacetylation of HSP90 and that this would destabilize AR and attenuate AR signaling. **Methods:** LNCaP and VCaP prostate cancer cells were treated with sulforaphane. Co-immunoprecipitation studies were performed to assess changes in acetylation of HSP90 and its interaction with AR after sulforaphane treatment. Western blots were performed to assess the effect of increasing doses of sulforaphane on AR protein expression and the effect of proteasome inhibition on sulforaphane treatment effects. QRTPCR was performed to determine whether sulforaphane resulted in reduced AR target gene expression, and ChIP was performed to assess whether enrichment of AR was decreased at its target genes. Sulforaphane's effects on HDAC6 enzymatic activity were assessed in an in vitro assay, and the consequences of overexpressing HDAC6 on sulforaphane treatment effects were also determined. Small interfering RNA against HDAC6 was transfected in order to compare this effect with sulforaphane treatment.
Results: Sulforaphane treatment increases the acetylation of HSP90, dissociating it from AR, and consequently leads to AR protein degradation, which is reversed by proteasome inhibition, diminished AR target gene expression, and reduced AR enrichment at its target genes. Sulforaphane also inhibits HDAC6 deacetylase enzymatic activity, and overexpression of HDAC6 attenuates sulforaphane-mediated AR protein depletion while small interfering RNA to HDAC6 recapitulates the sulforaphane treatment effects. **Conclusions:** The inactivation by sulforaphane of HDAC6-mediated HSP90 deacetylation and consequent attenuation of AR signaling represents a newly-defined mechanism that may help explain this agent's activity in prostate cancer.

311 Chemotherapy-Induced Monoamine Oxidase A Expression in Prostate Carcinoma Associates With Clinical Outcome, Functions as a Cytoprotective Resistance Enzyme, and May Serve as an Imaging Target in Advanced Disease

William Harris[1], Mengchu Wu[1], Chung-Ying Huang[1], Hong Gee Sim[1], Celestia S. Higano[2], Lawrence D. True[2], Robert Vessella[2], Paul H. Lange[2], Mark Garzotto[3], Tomasz M. Beer[3], Kenneth Krohn[2], Jeanne Link[2], **Peter S. Nelson**[1]

[1]Fred Hutchinson Cancer Research Center; [2]University of Washington; [3]Oregon Health and Sciences University

To identify molecular determinants of chemotherapy resistance, patients with high-risk localized prostate cancer (TNM > T2b or PSA >15 ng/ml or Gleason glade > 4+3) were enrolled into a phase II clinical trial of neoadjuvant chemotherapy with docetaxel and mitoxantrone followed by prostatectomy. Pre-treatment prostate tissue was acquired by needle biopsy and post-treatment tissue was acquired by prostatectomy. Prostate epithelium was captured by microdissection, and transcript levels were quantitated by cDNA microarray hybridization. Gene expression changes after chemotherapy were measured in 31 patients who completed four cycles of neoadjuvant chemotherapy. The transcript encoding monoamine oxidase A (MAOA) was significantly increased following chemotherapy and associated with subsequent biochemical relapse. In vitro, cytotoxic chemotherapy induced the expression of MAOA, and overexpression of MAOA led to resistance to docetaxel. Furthermore, inhibition of MAOA activity using the irreversible inhibitor Clorgyline augmented the apoptotic response induced by docetaxel. We further found that MAOA overexpressing cells have higher level of ROS as the byproduct of MAOA enzymatic activity. As a result, hypoxia induced factor 1 α (HIF1α) also increased significantly. As radiolabeled substrates of MAOA have been developed for positron emission tomography (PET) studies of brain metabolism, we explored the use of MAOA PET for imaging prostate tumors and found that xenografted prostate cancers were distinguished from background tissues by virtue of MAOA activity. In summary, these results indicate that MAOA functions as a cytoprotective resistance enzyme and contributes to docetaxel resistance through induction of the HIF1α pathway. As several MAOA inhibitors have already been approved for clinical use, exploiting their potential to enhance the effectiveness of therapies for prostate cancer should be evaluated.

312 Mayo Clinic SPORE in Ovarian Cancer

Lynn Hartmann, Scott Kaufmann

Mayo Clinic, Rochester, MN

Ovarian cancer has the highest mortality rate of the "women's cancers" (breast and gynecologic cancers). The Mayo Clinic Ovarian SPORE features a team of basic, clinical, and population science investigators conducting translational research designed to reduce the burden of ovarian cancer. The translational science of this SPORE includes four projects. Project 1, "Poisoning of PARP and topoisomerase I to treat ovarian cancer," studies the effect of the poly (ADP-ribose) polymerase (PARP) inhibitor ABT-888 alone and in combination with topotecan. In addition to elucidating how ABT-888 allows PARP to actively contribute to the demise of topotecan-treated ovarian cancer cells, this project will examine a series of tumor markers for their ability to predict response in a topotecan/ABT-888 phase II trial. Project 2, "Mechanisms of immunosuppression in ovarian cancer," studies the components of microenvironmental immunosuppression in ovarian cancer. Based in a large cohort of ovarian cancer cases, the work will examine differences in inherited variation in regulatory T-cell-related genes, Tregs, in the microenvironment and outcome after diagnosis. Project 3, "Optimizing measles virotherapy in the treatment of recurrent ovarian cancer," builds upon our in vitro and phase I clinical trial data showing promising anti-tumor activity with the attenuated vaccine strain of measles virus delivered intraperitoneally in women with recurrent ovarian cancer. This project will address the possibility of systemic administration of the virus and measures to enhance cytotoxicity. Project 4, "Flavopiridol reverses platinum resistance in ovarian cancer," builds upon work at Mayo showing that flavopiridol combines with cisplatin to yield cytotoxic synergy. We completed a phase I trial of this combination and now, in a phase II trial in platinum-resistant ovarian cancer, have observed a 33% response rate, which is twice the mean response rate typically seen in this setting. This project will study the mechanism of synergy and attempt to identify predictive biomarkers in samples from responders versus non-responders. Four cores (Administration, Biospecimens, Animal Models, and Biostatistics) provide infrastructure support. A Developmental Research Program will foster promising research initiatives, including extension of ongoing collaborations with TCGA and UCLA. A Career Development Program will promote the research careers of investigators who wish to pursue ovarian cancer translational research. Patient advocates will be involved with each project and will assist in prioritizing translational research.

313 Differential Expression of MicroRNAs in Tamoxifen-Sensitive Versus Tamoxifen-Resistant Human Breast Cancer Cells

Carolyn M. Klinge[1], Nalinie S. Wickramasinghe[1], Tissa T. Manavalan[1], Susmita Datta[2], Theodore S. Kalbfleisch[1]

[1]Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine; [2]Department of Bioinformatics and Biostatistics, School of Public Health and Information Sciences, University of Louisville

Select changes in microRNA (miRNA) expression correlate with diagnostic markers used in early stage breast cancer therapies (e.g., estrogen receptor alpha [ERalpha]). Comparatively little is known about miRNA regulation or how antiestrogens (e.g., tamoxifen [TAM]), regulate the expression of miRNAs in breast cancer cells. The goal of our research is to determine the identity and function of miRNAs whose expression is differentially regulated by estradiol (E2) and TAM in antiestrogen/tamoxifen-sensitive versus -resistant breast cancer cell lines and to correlate these miRs and their gene targets with those dysregulated in human breast tumors, thus offering new biomarkers to be tested in patient prognosis and treatment planning. We used miRNA microarray analysis to identify miRNAs differentially expressed and regulated by E2 and 4-hydroxytamoxifen (4-OHT) in MCF-7 tamoxifen-sensitive, estrogen-dependent versus LY2 tamoxifen/endocrine-resistant human breast cancer cells. Four separate experiments were performed for each treatment group. Bioinformatic analyses to impute the biological significance of the identified miRNAs by identifying their computationally predicted target genes in the human genome using TargetScan, PicTar, and the Sanger miRBase Targets databases were performed. Additionally, we compared global miRNA and mRNA expression patterns in 4-OHT-treated MCF-7 cells to identify key targets. We experimentally confirmed the observation that E2 reduced miR-21 expression in MCF-7 cells. This repression was inhibited by the antiestrogen ICI 182,780 (Faslodex) and ERalpha knockdown by siRNA, indicating that the E2-suppression is ERalpha-mediated. E2 increased luciferase activity from reporters containing the miR-21 recognition elements from the 3'-UTRs of miR-21 target genes, corroborating that E2 represses miR-21 expression resulting in a loss of target gene suppression. The E2-mediated decrease in miR-21 correlated with increased protein expression of endogenous miR-21-targets Pdcd4, PTEN, and Bcl-2. We are currently performing quantitative RT-PCR (Q-PCR) assessment of the miRNAs identified by microarray and examining changes in target protein expression by western blot analyses.

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314 Cyr61 a Key Target for Zometa (Zoledronic Acid) in Breast Cancer

Ruth Lupu, Ingrid Espiniza

Medicine Experimental Pathology, Mayo Clinic, Rochester, MN

CYR61 is a pro-angiogenic factor that is overexpressed in about 30% of breast carcinomas, most of which are triple negative carcinomas. Cyr61 binds to several integrins, one of which is αvß3. αvß3 expression is also overexpressed in a subset of aggressive breast carcinomas. Breast cancer cells that overexpress both CYR61 and αvß3 are triple negative breast cancer cell and are metastatic to the lung, liver, and bone. Therefore, CYR61 represents a good target for therapy and a good prognostic indicator. Women with advanced breast cancer often receive bisphosphonate treatment as a complementary treatment rather than as a chemotherapeutic one. Zometa® (Zoledronic acid: ZOL), a third generation amino-biphosphonate, inhibits bone resorption and might prevent development of new osteolytic lesions. It has been shown that CYR61 is expressed in 30% of triple negative breast carcinomas and that αvß3 is a poor prognostic indicator for invasive breast carcinomas. CYR61/αvß3 autocrine loop is involved in breast cancer cell survival, proliferation, and chemoresistance. ZOL inhibits αvß3 expression in the cell surface of endothelial cells, and CYR61 upregulates the αvß3 expression in breast cancer cells. However, whether CYR61 is affected by amino-biphosphonates is unknown. We tested the effect of ZOL in breast cancer cells overexpressing both CYR61 and αvß3 and found that the IC50 for Zometa® was μM as much lower than for cells which do not express CYR61. Interestingly, ZOL-inhibited CYR61 overexpressing cells anchorage independent growth as well as their ability to grow in 3D cell culture matrigel, namely ZOL-blocked CYR61 overexpressing cells branching and morphogenesis. Then, we tested whether the effect of ZOL was at the promoter transcriptional level. ZOL had a significant inhibitory effect on the transcription of CYR61 in a dose-dependent manner. Interestingly, we further demonstrated that deletion of FOXO3a site at the CYR61 promoter (CYR61-FOXO3a) prevents the effect of ZOL-induced inhibitory effect on CYR61 transcription activity. We also confirmed that ZOL decreased the levels of CYR61 protein expression. We show a direct effect of ZOL on CYR61 expression at the transcriptional and protein level. We have shown, for the first time, the specific effect of ZOL on an angiogenic factor involved in the metastatic effect of breast cancer cells, a finding that urges the development of a new line of therapy in metastatic endothelial cells.

315 Novel Radio-Viro Therapy for Prostate Cancer Using the Sodium-Iodide Symporter and 131-I: A Phase I Clinical Trial of Cancer Gene Therapy

John C. Morris, Torrence M. Wilson, Brian J. Davis, O. Kenneth MacDonald, Gregory A. Wiseman, Mark J. Federspiel, Eva Galanis, Donald J. Tindall

Departments of Urology, Oncology, Radiology, and Molecular Medicine and Division of Endocrinology, Mayo Clinic Prostate Cancer SPORE, Rochester, MN

Radioiodine (131I) is utilized routinely for therapy of recurrent and metastatic thyroid cancer because of the native expression of NIS in that tumor; our protocol seeks to bring this effective therapy to men with prostate cancer. Ad-CMV-NIS is an E1A-deleted, replication-deficient adenovirus containing the cDNA for the human sodium-iodide symporter (NIS) and the cytomegalovirus early gene promoter. The product of the NIS gene is the NIS protein, which is normally expressed by functioning thyroid tissues and thereby causes uptake and concentration of iodine. Infection of prostate cancer cells with Ad-CMV-NIS causes expression of the NIS protein, which induces high level uptake and concentration of iodine by the prostate cells. This induced iodine uptake allows imaging and therapy of the prostate cancer by administration of radioactive iodine as we have demonstrated in preclinical small and large animal models.

We have recently opened a phase I clinical trial of Ad-CMV-NIS. Viral particles are directly injected into the tumors of men with prostate cancer that is locally recurrent following external beam radiotherapy. The virus is injected trans-perineally thru needles inserted by trans-rectal ultrasound imaging, a method similar to that practiced for prostate brachytherapy. NIS expression, reflected by iodine uptake, is monitored and quantitated using SPECT-CT fusion imaging following 123I administration, and dosimetry is performed. A single therapeutic dose of 131I, the quantity determined by dosimetry measurements (maximum dose of 200 mCi), is to be administered to the subjects if threshold uptake is achieved to deliver between 5 and 20 Gy to the prostate. By our original design, four doses will be evaluated in up to 17 men beginning at 109 viral particles, and maximum dose will be 1012 particles, based upon tolerance, three patients per dose level. We are currently revising our protocol to an accelerated two-step design that will include only one patient at the lower doses if no dose-limiting toxicity is observed. The patients will be followed by PSA measurements and imaging for response determination of the MTD utilizing standard endpoints. At the time of writing of this abstratct, one patient has been completed and no toxicity has been observed.

316 Signaling and Progression in Prostate Cancer

Bryce Paschal, **Dan Theodorescu**

University of Virginia, Charlottesville

Metastatic, hormone independent prostate cancer (CaP) is incurable. The goal of this multidisciplinary program project is to elucidate the signal transduction mechanisms that underlie the stepwise events associated with progression of CaP from a localized and androgen sensitive tumor to a disseminated and androgen independent one. The program brings together productive and experienced investigators with complementary expertise relevant to the stated goal of the program and backgrounds in signal transduction (J. T. Parsons, S. J. Parsons, Weber), nuclear receptor biology (Paschal), bone biology (Guise), human prostate cancer pathology (Frierson), biostatistics (Conaway), and basic and clinical prostate cancer metastasis research (Theodorescu). In Project 1, Theodorescu and J. T. Parsons propose to evaluate the roles of VEGF, FAK, and Rap in CaP progression and metastasis to bone. In Project 2, S. J. Parsons studies the regulation of neuroendocrine cell growth within advanced prostate cancers and the impact of such cells on overall tumor dependence on androgen. In Project 3, M. Weber studies Ras-mediated signaling cascades as they affect ligand hypersensitive androgen receptor activity. In Project 4, Paschal proposes to study the relationship between androgen receptor activation and the control of its nuclear localization. This interactive program relies heavily on synergistic technical and scientific expertise from all investigators. The productivity of individual projects is catalyzed by highly interactive cores. Led by Theodorescu, Administrative Core A integrates the participation of M. Conaway, an expert biostatistician. Core B, Cell, Animal and Imaging, is led by Guise, who has extensive experience in bone histology and histomorphometry and is familiar with the biology of prostate cancer and the xenograft models used in prostate cancer research as well as their in vivo imaging. Frierson, an expert surgical pathologist who specializes in CaP, leads Tissue Analysis Core C. Together, these projects and cores integrate diverse skills and expertise to focus on areas fundamental to our understanding of tumor progression in CaP, with the objective of accelerating progress in developing a cure for this devastating disease.

317 Targeting the Six1-Eya Transcriptional Complex for Anti-Breast Cancer Therapy

Aaron Patrick[1,2], Aaron Krueger[1], Douglas Micalizzi[3,4], Erica McCoy[4], Rui Zhao[1,3,4], **Heide L. Ford**[1,2,3,4]

[1]Department of Biochemistry and Molecular Genetics, [2]Department of Ob/Gyn, Division of Basic Reproductive Sciences, [3]Medical Scientist Training Program, [4]Program in Molecular Biology, University of Colorado Denver, School of Medicine

Six1 is a homeoprotein with limited expression in adult tissues and frequent misexpression in human malignancies. We have recently demonstrated that misexpression of Six1 in mouse mammary gland epithelium induces mammary tumors of multiple histological subtypes in which the majority of tumors undergo an epithelial-to-mesenchymal transition and exhibit a stem cell phenotype. We have further demonstrated, using mouse metastasis models, that Six1 overexpression also induces breast cancer metastasis. Importantly, breast cancer patients whose tumors overexpress Six1 had a shortened time to relapse and metastasis and an overall decrease in survival. Because Six1 does not have an intrinsic activation domain, it requires the Eya family of co-activators to activate transcription. Indeed, the Eya proteins utilize their intrinsic phosphatase activity to switch the Six1 transcriptional complex from a repressor to an activator. The Six1-Eya interaction is essential for proliferation during embryonic development, and both Six1 and Eya2 have been implicated in the same types of cancer. We have shown that coordinated overexpression of the two genes increases the significant correlation with shortened time to relapse and metastasis and with decreased survival in breast cancers. These findings suggest that Eya2 and Six1 cooperate to stimulate breast tumor progression. Because the Eya co-activator contains two unique protein phosphatase domains, it may serve as a novel anti-cancer drug target along with Six1. Thus, we are testing the hypothesis that the Six1/Eya/DNA complex is an ideal drug target whose inactivation will inhibit tumor cell proliferation, survival, and metastasis in breast and possibly other cancers. To accomplish this goal, we are using a combination of X-ray crystallography, for future structure-based rationale drug design, and high throughput screening (HTS), to directly identify small molecule inhibitors of the Six1/Eya transcriptional complex. We have obtained small crystals of the Six1/Eya2 Eya domain complex and collected a 4Å diffraction data set. We also have begun virtual small molecule screening using the recently solved Eya domain structure. In addition, we are developing HTS assays to screen small molecules against the Six1/DNA and Six1/Eya interaction, Eya's phosphatase activity, and Six1's transcriptional activity using a luciferase assay. Progress from both the virtual and high throughput screens will be discussed.

318 A Functional Genomics Approach to Therapeutic Discovery in Human Breast Cancer

Earlene Schmitt[1,2], Ronald J. Bernardi[3], Ji Luo[4], Kris Kahle[4], Chad A. Shaw[1], Rachel Schiff[5], C. Kent Osborne[5], Stephen J. Elledge[4], **Thomas F. Westbrook**[1,2]

Departments of [1]Molecular & Human Genetics and [2]Biochemistry & Molecular Biology, Baylor College of Medicine, Houston, TX; [3]Department of Pediatrics, Texas Children's Cancer Center and Baylor College of Medicine, Houston, TX; [4]HHMI and Department of Genetics, Harvard Medical School, Boston, MA; [5]Department of Medicine and Lester & Sue Smith Breast Center, Baylor College of Medicine, Houston, TX

Discovering genotype-selective therapeutic targets: Our research program is dedicated to developing new approaches for rapidly expanding the number of therapeutic targets for human breast cancer. The major goal for any cancer therapy is to selectively kill tumor cells without detrimental effects on normal tissues. Implicit in this goal is the challenge that therapies must target vulnerabilities unique to cancer cells. To address this challenge, the breast cancer community has largely focused on targeting cancer-causing oncogenes. For example, Herceptin and other HER-family inhibitors have been effective in combatting HER2-positive breast cancers. However, many prevalent oncogenes (such as c-Myc) have proven difficult to inhibit pharmacologically. Thus, we need new ways to identify therapeutic targets for cancers driven by these oncogenes. In addition to promoting tumorigenesis, oncogenes also produce unique stresses in cancer cells, collectively termed "oncogenic stress." Consequently, cancer cells become dependent on pathways enabling them to tolerate this stress. For example, some oncogenes confer an increased dependence on the heatshock response and aerobic glycolysis, two processes that have recently received therapeutic attention. These "stress-support pathways" would be ideal therapeutic targets because cancer cells (but not normal cells) become hyper-dependent on them for their growth and survival. However, pathways underlying these processes are largely unexplored for two reasons: (1) it is difficult to predict which genes are involved in stress support for a given oncogene or tumor type; and (2) stress-support genes may not themselves be oncogenes or otherwise mutated in cancer, and thus cannot be discovered directly through analysis of cancer genomes / epigenomes. Therefore, identification of stress-support pathways requires unbiased, functional approaches. We have developed new whole-genome RNA interference (RNAi) technologies that enable such a functional approach. We are using these unique genetic technologies to systematically discover oncogene-specific stress support pathways, thus rapidly expanding the number of potential therapeutic targets. These new stress support pathways are being tested by integrative in silico, in vitro, and in vivo approaches to define promising targets for therapeutic development. This strategy is focused on identifying the genes supporting aggressive breast cancers driven by common oncogenes such as c-Myc.

319 Prevention of NNK-Induced Lung Cancer in Mice by the Aromatase Inhibitor Anastrozole

Jill M. Siegfried, Mary Rothstein, Stephanie Land, Laura P. Stabile

University of Pittsburgh Cancer Institute, Pittsburgh, PA

Estrogens are known to be involved in lung cancer proliferation and progression. Lung tumors have previously been shown by us and others to express the enzyme aromatase and to contain physiologic levels of beta-estradiol. We have also shown that lung tumors overexpress one of the estrogen receptors, ERbeta, as well as the estrogen-binding protein GPR30, which is also thought to transduce estrogenic signals. Lung tumors therefore often contain an autocrine system of producing and responding to beta-estradiol, and this autocrine system may be upregulated compared to normal lung. We have previously shown that interruption of estrogen signaling by downmodulating estrogen receptors with the anti-estrogen fulvestrant results in decreased human lung tumor growth in a xenograft model and that addition of the aromatase inhibitor anastrozole to fulvestrant results in a greater degree of apoptosis in human lung tumor xenografts. To determine whether an aromatase inhibitor could also prevent the development of lung cancer induced by a tobacco carcinogen, we exposed ovariectomized female mice in a susceptible strain (FVB/N) to 24 mg of the tobacco carcinogen NNK from weeks 1–4. All mice received daily supplementation with 0.1mg androstenedione (s.c.), the substrate for aromatase, beginning at week 2. Anastrozole at 0.1mg/k (n=11) or placebo (n=10) was administered by oral gavage daily from week 2 until week 15, at which time mice were sacrificed and lung tumors were evaluated. A highly significant decrease in the number of lung tumors per animal was observed with anastrozole treatment (mean 1.3, range 0–2), compared to placebo (mean 3.3, range 0–7, P = 0.003, Poisson regression). There was also a significant decrease in mean tumor size with anastrozole (mean 0.09 mm2) compared to placebo (mean 0.25 mm2, P = 0.001, mixed effects modeling). Ki67 labeling, p-MAPK, and aromatase were also evaluated. Aromatase and P-MAPK are expressed in tumors from placebo and anastrozole groups to the same extent, while Ki67 expression is decreased in anastrozole treated tumors. We now plan to combine anastrozole with fulvestrant in the chemoprevention protocol to determine whether a further decrease in lung tumor formation can be induced by blocking both estrogen production and ER signaling. These results suggest that aromatase inhibitors can block lung tumor formation as well as reduce growth of established tumor xenografts. Modulating estrogen signaling by blocking aromatase alone or in combination with blocking estrogen receptors might be useful for lung cancer therapy and lung cancer prevention.

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320 Prolongation of Off-Cycle Interval by Finasteride Is Not Associated With Survival Improvement in Intermittent Androgen Deprivation Therapy in LNCaP Tumor Model

Yujuan Wang[1], Shubham Gupta[1], Vi Hua[2], Raquel Ramos-Garcia[1], Daniel Shevrin[3], Borko D. Jovanovic[4], Joel B Nelson[1], **Zhou Wang**[1]

[1]Department of Urology, University of Pittsburgh, Pittsburgh, PA; [2]Department of Urology, 4Department of Preventive Medicine, Northwestern University, Chicago, IL; [3]NorthShore University Health System Medical Group, Evanston, IL

We have previously reported that finasteride administration in intermittent androgen deprivation therapy (IADT) can improve survival of nude mice bearing LNCaP xenograft tumors when the duration of off-cycle in IADT was fixed. A recent retrospective study showed that addition of finasteride doubled the duration of the off-cycle, without changing progression to castration resistance. In view of the above difference, we attempted to investigate the relationship of 5alpha-reductase inhibition with the off-cycle interval and overall survival in a murine model.

Subcutaneous LNCaP tumors were established in nude mice (Balb/C-Nu). After the tumors reached a size of 0.5 cm in diameter, the mice were castrated and followed up for 2 weeks, after which they were randomized to continuous androgen deprivation (CAD), CAD plus finasteride, IADT, and IADT plus finasteride. The off-cycle was discontinued when the tumor volume was doubled. Subsequent cycles were carried out similarly. Our study showed that use of finasteride during the off-cycle of IADT doubled the first off-cycle duration. However, prolongation of the off-cycle by finasteride did not translate into an increase in overall survival.

Our findings suggest that the survival advantage of IADT+F over IADT that we previously reported was lost when the off-cycle prolongation by finasteride was allowed. Thus, we conclude that maximum possible lengthening of the off-cycle by 5alpha-reductase inhibition is not associated with survival improvement in an LNCaP xenograft model.

321 Anti-DR5 Antibody Therapy for Triple Negative Breast Cancer

Tong Zhou[1], Donald J. Buchsbaum[2], Albert F. Lobuglio[3]

Comprehensive Cancer Center, University of Alabama at Birmingham

Triple negative breast cancer (TNBC) represents a significant proportion of breast cancer patients (20–25%) and has a poor prognosis, and no targeted approach to therapy has been found; thus, new agents or combination of agents are needed. Ten years ago, we developed an agonistic anti-DR5 antibody, TRA-8, which in pre-clinical studies exhibited a strong apoptosis-inducing activity and anti-tumor efficacy against a variety of tumor cell lines. Tumor cell lines of each tumor type were heterogeneous in their sensitivity to TRA-8. In tumor cell lines that were sensitive to TRA-8, the combination of TRA-8 plus chemotherapy had synergistic or additive enhancement of cytotoxicity over either agent alone. In collaboration with our industry partner, Daiichi Sankyo Co. Ltd., we have carried out a Phase I trial of CS-1008 (humanized version of TRA-8) in patients with refractory malignancies. CS-1008 was well tolerated at doses as high as 8 mg/kg weekly x 6 (48 mg/kg total dose). Recently, we found that basal-like genotype breast cancer cell lines were sensitive to TRA-8 mediated cytotoxicity, while luminal genotype cell lines were consistently resistant. Further, the anti-tumor efficacy of TRA-8 and TRA-8 chemotherapy to a basal-like breast cancer cell line (2MLP) far exceeded that seen with other tumor cell types. In searching the signaling mechanisms by which TNBC exhibits increased susceptibility to DR5-mediated apoptosis, we found that DDX3, a member of the DEAD Box protein family, abnormally associated with DR5 in all TNBC cell lines. As a novel death receptor-associated adapter protein, DDX3 could associate with a non-death domain region of the cytoplasmic tail of DR5 and recruit cIAP1 via its Caspase Recruiting Domain (CARD), thereby forming an inhibitory complex of DR5/DDX3/cIAP1 to antagonize the death domain function in the majority of DR5-apoptosis resistant cell lines. The levels of cIAP1 and other apoptosis inhibitory proteins in the DR5/DDX3 complex in TNBC were significantly lower than those of DR5-resistant cells. The downmodulation of IAPs in TNBC cells by a small molecule IAP inhibitor further enhances TRA-8-mediated apoptosis of TNBC cells. Thus, we believe that DR5 represents an attractive target on basal-like breast cancer cells and can be exploited together with chemotherapy as a new therapeutic strategy for TNBC.

322 A Screen of the NCI Natural Products Repository for Inhibitors of Tyrosyl-DNA-Phosphodiesterase I (Tdp-1)

Alun Bermingham[1], Edmund Price[1], Thomas Dexheimer[2], Christophe Marchand[2], Jason R. Evans[1], Ekaterina Goncharova[1], James B. McMahon[1], Yves Pommier[2], **Barry R. O'Keefe**[1]

[1]Molecular Targets Development Program, Center for Cancer Research, National Cancer Institute-Frederick, Frederick, MD; [2]Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, Bethesda, MD

Tyrosyl-DNA-phosphodiesterase I (Tdp-1) is part of the DNA repair complex that resolves irreversible topoisomerase I (Top1)-DNA cleavage complexes by catalyzing the hydrolysis of 3'-phosphotyrosyl bonds. A point mutation in the Tdp1 gene causes a neurological disorder called spinocerebellar ataxia with axonal neuropathy (SCAN1) (Takashima, 2002). SCAN1 cells display hypersensitivity to camptothecin (CPT), a potent Topoisomerase 1 inhibitor. Additionally, overexpression of a human Tdp1 fusion protein alleviates some of the effects of CPT treatment (Barthelmes, 2004). These observations suggest that inhibitors of Tdp1 could act synergistically with CPT in a combined therapeutic treatment for certain cancers. Despite attempts to identify synthetic small molecule inhibitors of Tdp-1, there are few verified inhibitors of this enzyme activity and none currently in clinical trials. While previous screening efforts have focused mainly upon compounds of synthetic origin (Antony, 2007), little effort has been directed toward the discovery of natural product derived inhibitors. The Molecular Targets Development Program (MTDP) has developed a fluorescence-based assay for the rapid identification and characterization of natural product derived inhibitors of Tdp1 enzyme activity. Initial high-throughput hit identification work has been carried out and a number of extracts displaying Tdp1 inhibition identified. Initial screening work is currently being followed up by hit evaluation of active compounds and bioassay guided fractionation of extracts. Selected purified compounds will then be pursued further by functional analysis in biological systems in the Laboratory of Molecular Pharmacology (LMP).

323 A Phase I Study of Nelfinavir, an FDA-Approved HIV Protease Inhibitor, in Adults With Solid Tumors

Gideon Blumenthal[1], Wendy Bernstein[1], Marc Ballas[1], Hyejeong Root[1], Erin R. Gardner[2], Shigeru Kawabata[1], Betsy Morrow[1], Jaclyn LoPiccolo[1], Cynthia Helsabeck[1], William D. Figg[1], **Phillip A. Dennis**[1]

[1]Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD; [2]Clinical Pharmacology Program, SAIC-Frederick, National Cancer Institute-Frederick, Frederick, MD

Background: Preclinical studies show that HIV protease inhibitors such as nelfinavir (N) have dose-dependent, pleiotropic, anti-cancer activities. However, the maximum tolerated dose (MTD) of N has not been established in humans.

Methods: Subjects with refractory metastatic cancer were treated on a modified Fibonacci dose-escalation scheme with a twice daily oral dose of N starting at the FDA-approved dose of 1250 mg bid on a 21-day cycle. Subjects with refractory solid tumors, ECOG PS < 2, and adequate organ function were eligible. Therapy continued until MTD or disease progression. PBMCs as well as optional tumor biopsies were collected for Akt inhibition and expression of markers of ER stress (ERS).

Results: Twenty-two subjects have been enrolled. Of 19 evaluable for toxicity, there were 16 men, 14 Caucasians, and 2 African Americans (median age 63 years (range 25–85)). Tumor types include lung (6) colorectal (4), thyroid (3), adenoid cystic (2), pancreatic (1), carcinoid (1), prostate (1), and renal cell (1). Median number of prior systemic therapies was 2 (range, 1–7). Dose levels were 1250 po bid (n=3), 1875 po bid (n=3), 2500 po bid (n=3), 3125 po bid (n=6), and 3750 po bid (n=3). N has been well tolerated with the most frequent non-hematologic Gr 1 and Gr 2 toxicities of diarrhea (39%), hyperglycemia (22%), and increased ALT (22%), with single episodes of Gr 3 diarrhea (n=1), hypothyroid (n=1), hypotension (n=1), and hypophosphatemia (n=1). At 3750 mg bid, there were two dose-limiting toxicities of Gr 4 neutropenia that were rapid in onset and rapid in resolution after discontinuation of N. Morphological evidence of N-induced ER stress was evident in peripheral blood smears from these subjects. There have been no responses. Five subjects (3/6 at DL4) have had stable disease for >12 weeks. Preliminary pharmacokinetic data revealed large inter-subject variability with a linear increase in exposure (AUC) at steady state. Steady state concentrations achieved at DL2 or above were active in preclinical studies. Akt inhibition and increased expression of markers of ERS have been observed in PBMCs from week 1 in the majority of patients at every dose level, but there was no correlation with clinical response.

Conclusions: N appears to be well tolerated in subjects with advanced solid tumors. The MTD is 3125 mg bid. This study continues to accrue at the MTD for further evaluation of efficacy, pharmacokinetics, and biomarker modulation.

324 Effective Treatment of Metastatic Insulinoma Using Multiple Treatments With Systemic RNA Interference Targeting Mouse PDX-1 In A Mouse Insulinoma Model

Francis Charles Brunicardi, Shi-He Liu, Min Li, Nancy Smyth Templeton, William Fisher

Michael E. DeBakey Department of Surgery, Houston, TX

Introduction: PDX-1 has been shown to be a potential molecular target for insulinomas. The purpose of this study was to determine whether multiple treatments with iv liposomally-delivered small hairpin-RNA targeting PDX-1 (PDX-1 shRNA) could serve as PDX-1-based therapy for metastatic insulinoma in a SCID mouse model.

Methods: Mouse PDX-1 shRNA (mPDX-1 shRNA) was cloned into pSUPER vector. The efficiency of silencing mPDX-1 gene expression and the ability of inhibitory cell proliferation in vitro was determined by western blot and MTS assay, respectively, after mPDX-1 shRNA transfection of mouse insulinoma (βTC-6) cells. An IP insulinoma mouse model was created and grouped to receive 1st : one treatment cycle, 2nd : two cycles and 3rd : three cycles of biweekly, iv liposomally-delivered mPDX-1 shRNA (L-mPDX-1 shRNA) or corresponding liposomally-delivered pSUPER as control via tail vein injection, (n=5-10 mice/group). Immunohistochemistry studies and TUNEL assay were performed on tumor and pancreas sections. Serum glucose and insulin levels were measured biweekly. Survival was analyzed by Kaplan-Meier in SPSS statistical software.

Results: In vitro knockdown of PDX-1 expression in cells was consistent with significant inhibition of cell proliferation as determined by MTS, showing 2-fold and 2.5-fold reduction of cell proliferation as compared to control at 48 and 72 h after shRNA transfection, respectively($p<0.05$). All three in vivo treatment cycles of iv L-mPDX-1 shRNA resulted in reduced insulin levels and increased glucose levels in the β TC-6 SCID mice compared to controls ($p<0.05$ for 2nd and 3rd cycles). Both insulin and glucose levels returned to normal in survival mice at 6 months after treatment. All treated mice had prolonged survival compared to controls (53.0±1.5d); however, the survival time in 3rd group mice (180±39.4d; all mice in this group survived and were sacrificed) was longer than that of 2nd (123.0±13.1d) and 1st group (129±38.5d) ($p<0.05$). Islet histology revealed decreased PDX-1, insulin, and PP expression and increased SST expression. Islet cell apoptosis was increased in all three treatment groups.

Conclusion: Multiple treatments of iv L-PDX-1 shRNA were effective against metastatic insulinoma in a SCID mouse model. The therapy prevented hyperinsulinemic death; however, it resulted in a mild form of type 3 diabetes due to PDX-1 knockdown effects on islet cells and apoptosis of the endocrine pancreas, which was reversible 6 months after treatment. This study demonstrates that PDX-1 targeting therapy is feasible and translational for treatment of insulinoma.

325 Combined Modality Targeted Therapy of Pancreatic Cancer With Anti-DR5 Monoclonal Antibody

Donald J. Buchsbaum, Tong Zhou, Patsy G. Oliver, Andres Forero, Albert F. LoBuglio

University of Alabama at Birmingham

We have developed an anti-DR5 monoclonal antibody therapy for pancreatic cancer through in vitro studies, animal model efficacy studies, and with an industry partner (Daiichi Sankyo), Phase I and recently completed Phase II trials in pancreatic cancer. We have also discovered a novel DR5 associated molecule (DDX3) that appears to play a major role in regulation of anti-DR5 mediated apoptosis by recruitment of IAPs to the DR5/DDX3 complex. We have been exploring a strategy to enhance the efficacy of anti-DR5 monoclonal antibody treatment especially against pancreatic tumor cell lines that have intermediate sensitivity or are resistant to anti-DR5. There are two prevailing mechanisms thought to be responsible for tumor cell resistance to DR5-mediated apoptosis. The first is the above DR5/DDX3/IAP complex described by our group. The second is the DR5-mediated activation of NFκB with resultant elevation of Mcl-1 (Bcl-2 family member). Our strategy to enhance anti-DR5-mediated anti-tumor efficacy has been to utilize small molecule inhibitors of Bcl-2 and IAP family members to counteract the molecular mechanisms of resistance. We have an active collaboration with Ascenta Therapeutics, which provides us with AT-101 (Bcl-2 family inhibitor) and AT-406, an IAP family inhibitor for our in vitro and in vivo studies. This strategy is being studied using in vitro analysis of DR5-mediated cytotoxicity, molecular analysis of the apoptotic pathway, and animal models of orthotopic and metastatic tumors. This will lead to a future Phase I/II clinical trial of this strategy. In addition, we are continuing studies of DDX3 and the DR5/DDX3 complex as a putative biomarker for tumor cell sensitivity/resistance to death receptor mediated anti-tumor efficacy and as the target of enhancement strategies for anti-DR-mediated anti-tumor effects.

326 Completion of Preclinical Studies and Advancement of the Chimeric HSV, C134, for Phase I Clinical Trial

Kevin A. Cassady[1], David Bauer[2], Gregory Friedman[1], James Markert[2], G. Yancey Gillespie[2]

Departments of [1]Pediatrics and [2]Neurosurgery, University of Alabama in Birmingham

The chimeric HSV, C134, is a novel Δγ134.5-based oncolytic HSV that is capable of improved late viral protein synthesis and viral replication in tumor while maintaining the avirulence of a Δγ134.5 vector. Previous studies have shown that C134-treated animals show improved survival and tumor reduction over non-chimeric Δγ134.5 recombinants in both xenogeneic human gliomas and syngeneic murine gliomas implanted orthotopically (Shah 2007). A principal advantage of the C134 chimeric HSV over Δγ134.5 in brain tumor therapy is its improved replication and direct oncolytic activity in the tumor. Here, we present data that the chimeric HSV C134 is more effective than Δγ134.5 in additional tumor models tested. The virus infects, replicates, and spreads in CD133+ human glioma xenografts better than did several Δγ134.5 viruses. It also improved survival of nude mice implanted with a glioma cell line (D54MG) in which C134 did not possess a significant in vitro replication advantage (<1 log) over Δγ134.5 viruses. Finally, we show that the virus is safe in additional neurotoxicity studies (IHC, virus recovery) in mice as well as in the highly sensitive non-human primate, Aotus nancymae. These studies confirm that the C134 recombinant overcomes the major limitation of inadequate late gene expression characteristic of Δγ134.5 HSVs, resulting in a markedly improved oncolytic HSV. These studies provided sufficient proofs of efficacy and neurosafety in two animal models such that the RAID program has approved production and certification of cGMP C134 necessary for FDA and IND approval. When RAID-produced and qualified C134 becomes available, we will proceed with plans to conduct phase I clinical testing in patients with recurrent anaplastic gliomas.

327 Studies of PHY906, A Chinese Medicinal Formula, as Adjuvant Therapy for Cancer Patients Under Chemotherapy

Yung-Chi Cheng

Yale University School of Medicine, New Haven, CT

PHY906, a four-herb Chinese Medinical Formula first described 1800 years ago for the treatment of diarrhea, vomiting, nausea, intestinal cramps, and fever was found to enhance the antitumor activity of chemotherapeutic drugs with a variety of mechanisms of action and decrease global toxicity of Irinotecan in tumor-bearing mice. Deletion of any one of the four herbs in the formula will not have the same effect. PHY906 could be manufactured consistantly under GMP, judged by chemical and biological multiplex fingerprint (Phytomics) and analyzed by Phyto Similarity Index (PSI) Software. The first phase I/2a studies in combination with Irinotecan for the treatment of colon carcinoma showed no change of PK of Irinotecan and decreased diarrhea, vomiting, and nausea side effects in patients. Not all the chemicals from PHY906 could be detected in plasma from patients. Animal studies indicated that PHY906 could decrease the inflammatory partly by inhibition of iNOS and COX-2 enzyme activity and increase the recovery of damaged intestine by potentiating the Wnt pathway in stem/progenitor cells in Irinotecan-treated tumor-bearing mice. In addition, PHY906 could enhance apoptosis of tumor and decrease the imflamatory cytokines in plasma of Irinotecan-treated tumor-bearing mice. The chemicals from PHY906 involved are not the same for the multiple sites of action. The second phase I/2a studies in combination with Captocitabine for the treatment of unresectable Child Pough A hepatocellular carcinoma (HCC) indicated that there is no PHY906-associated toxicity and no Grade 3 and Grade 4 toxicity associated with treatment. The median survivial time of 20 patients was 10.9 months. This is equivalent to Sorafenib, the only approved HCC drug in US/Europe Phase 3 trial, but is much longer than Asian trials. The third phase of I/2a trial is to examine whether PHY906 could increase the maxium tolerated dosage of Captocitabine in advanced pancreatic carcinoma patients. The results indicated it is possible to escalate Captocitabine 150% without more toxicity. Phase II studies are on going. In conclusion PHY906 has the potential to enhance antitumor activity and decrease G.I.-related toxicity of a variety of chemotherapeutic agents in patients with cancer.

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328 Targeted Pre-Micro RNA Profiling for the Stratification of Viral-Associated Cancers That Benefit From Combination Anti-Viral/Anti-Cancer Therapy

Dirk P. Dittmer, Pauline Chugh

Lineberger Comprehensive Cancer Center, Center for AIDS Research, Department of Microbiology and Immunology, University of North Carolina at Chapel Hill

Twenty percent of cancers are causally linked to human tumor viruses. At present they are treated no different than cancers that are not viral associated. Yet, anti-viral discovery and development comprises a major focus for private and academic research. We hypothesize that patients with certain virally associated cancers would benefit from combined anti-viral/anti-cancer therapy, and we have identified biomarkers (and corresponding high-throughput assays) to identify these patients.

Micro RNAs represent a novel class of transcription products that are more stable than messenger RNAs. Micro RNAs are made from pre-micro RNAs. Pre-micro RNAs (and mRNAs) are also encoded by tumor viruses, and their transcription is correlated with distinct stages in the viral life cycle. In case of human herpes viruses, the presence of specific pre-micro RNAs signifies lytic viral replication, which is associated with the expression of viral thymidine kinases. Viral thymidine kinases convert the acyclovir/gancilovir family drugs into their active form, which inhibits viral and cellular DNA replication. These drugs are already FDA-approved and have a therapeutic index of >50.

We have shown in culture, in xenograft models, and in clinical case studies that, when combined with conventional cytotoxic drugs, these anti-viral drugs act synergistic but only for those cancers that express a “replicative” viral transcription profile.

Since only the cancer cells carry the virus, both the biomarker and partnered drug are highly specific.

329 Maximal Power Design and Analysis of Drug Synergy in Therapeutic Development: Applications to Vorinostat With Cytosine Arabinoside and Etoposide and Other Combination Studies

Hong-Bin Fang[1], Douglas D. Ross[1], Martin J. Edelman[1], Edward A. Sausville[1], Wei Li[2], **Ming T. Tan**[1]

[1]University of Maryland Greenebaum Cancer Center; [2]Department of Pharmaceutical Sciences, University of Tennessee Health Sciences

Drug combinations are the hallmark of cancer therapy. Preclinical experiments on multi-drug combinations are important steps to bring the therapy to clinic. A statistical approach for evaluating the joint effect of the combination is necessary because even in vitro experiments often demonstrate significant variation in dose-effect. Such variation needs to be accounted for in the experimental design and analysis. Our research has developed a maximal power (MP) design and interaction index surface (IIS) analysis methods for in vitro and in vivo (e.g., xenograft) combination studies so that the joint effect of a combination can be estimated efficiently and the most synergistic combination can be identified. We demonstrate that these statistical methods and software have resulted in the identification of highly synergistic dose combinations that could have been missed with classic methods.

The first study is the combination of vorinostat (suberoylanilide hydroxamic acid) combined with ara C and with etoposide in leukemia cell lines. The doses in the experiment were generated by the MP design and the data analyzed using the IIS approach so that synergistic, additive, and antagonistic interaction dose regions are identified. Cytotoxic antagonism resulted when vorinostat was combined concomitantly with ara-C; however, when vorinostat was given first, followed by a drug-free interval before ara-C treatment, this sequential combination was mostly synergistic. Etoposide combined with vorinostat was additive to synergistic, and the synergism became more pronounced when etoposide was given post a drug-free interval after vorinostat. These findings are used in designing the CTEP trial (NCI 6829: Phase I trial of vorinostat in combination with cytarabine and etoposide in patients with advanced acute leukemia and high-risk myelodysplastic syndromes, PI: Gojo/Ross). The interim results on toxicity and response have been consistent with the model.

Another study utilized a novel thiazolidine compound plus Sorafnib, where initial experiments using classic methods failed to identify synergistic combinations. Subsequent experiments using the MP methodologies demonstrated significant synergistic drug combinations. The SynStat R program for the design and analysis of drug synergy is available at http://www.umgcc.org/research/biostat_software.htm.

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330 Marine Microbial Metabolites Provide Access to New Drug Discovery Targets

William Fenical, Paul R. Jensen

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography; Moores Comprehensive Cancer Center, University of California, San Diego

Exploration of deep ocean sediments has provided access to an unprecedented group of microorganisms that provide considerable potential in the treatment of cancer. Marine actinomycete bacteria, in saltwater-based culture, have been found to produce an array of structurally-diverse molecules, many of which inhibit cancer cell replication by unusual mechanisms of action. The obligate marine actinomycete Salinispora tropica has yielded salinosporamide A, a unique beta-lactone that shows potent inhibition of the function of the 20S proteasome. The molecule exhibits picomolar activity against the chymotrypsin-like enzymatic function of the proteasome and significant antiproliferative effects in various cancer cell lines at the low nanomolar level. Salinosporamide A, under the name NPI-0052, was developed by Nereus Pharmaceuticals in San Diego, which has taken this unique compound to phase II human trials. In studies with the multiple myeloma (MM) drug Velcade™, salinosporamide A was found to outperform velcade and to illustrate its effects against Velcade™-resistant MM. A similar discovery of the compound halimide facilitated Nereus to develop a close analog, NPI-0058, which appears to be a selective inhibitor of tumor vascular epithelial cell division. NPI-0058 is currently in phase II with the anticipated targets breast and ovarian cancer. Rather than first defining targets and screening for inhibitors, this project examines drug quality leads and then elucidates their intracellular protein targets by fluorescent labeling, immunoprecipitation, and protein sequencing by mass spectrometry. This approach has resulted in specific compounds that target common intracellular targets such as tubulin and actin. However, this approach has also illustrated new targets potentially defining new approaches to cancer drug discovery. The ammosamides, alkaloids from a marine streptomycete target myosin II, an important intracellular protein involved in cytokinesis. The naphyradiomycins are a group of mixed biosynthetic compounds that inhibit GRP-94, a chaperone protein of the HSP-90 class of heat shock proteins. Overall, this approach is highly complementary to "known target" drug discovery and promises to continue to yield novel clinical candidates for the treatment of cancer.

331 Successful Developmental Pathway of Delta-24-RGD

Juan Fueyo, Marta Alonso, Raymund Yong, Candelaria Gomez-Manzano, W.K. Alfred Yung, Charles Conrad, Frederick Lang

The University of Texas M. D. Anderson Cancer Center

Malignant brain tumors are among the most deadly human cancers, with a median survival of only one year. New therapies are therefore urgently needed. We have shown that Delta-24-RGD, a replication-competent oncolytic virus characterized by our group at M. D. Anderson may be an effective treatment against gliomas. After showing that Delta-24-RGD treatment eradicated human gliomas in an animal model of the disease, we have initiated a Phase I clinical trial in patients with recurrent malignant gliomas (PI: FF Lang). Data from the first patients are indicating that Delta-24-RGD is a safe anti-glioma therapy. In addition, we aim to obtain information about the extent to which Delta-24-RGD is capable of producing oncolysis in tumors in patients. Consequently, a major goal of this project is to determine the potency with which Delta-24-RGD is capable of replicating in and spreading through human gliomas in situ. Although preclinical studies indicate successful viral replication and spread is likely to occur in patients' tumors, it is possible that for gliomas in situ complete tumor eradication (cure) may be limited at least partly by molecular impediments to viral oncolysis that reduce efficacy and by complex physical barriers that will reduce the spread of virus from the site of injection to the edges of these infiltrative brain tumors. In this context, we have shown that the efficacy of Delta-24-RGD is increased by combining it with specific chemotherapeutic agents, particularly Temozolomide (TMZ), the standard first-line therapy for gliomas. Interestingly, preliminary data indicate that Delta-24-RGD and TMZ have a synergistic anti-glioma effect, which is mediated at least in part by Delta-24-RGD-induced inhibition of MGMT and other DNA-repair pathways. In addition, the efficacy of the Delta-24-RGD can be improved by combining its administration with Temozolomide. Finally, we also propose a method to efficiently deliver Delta-24-RGD systemically. We hypothesize that packaging Delta-24-RGD within hMSCs will allow for systemic delivery of Delta-24-RGD in vivo, resulting in a significant anti-glioma effect. To test this hypothesis, in vivo studies are being undertaken to demonstrate the extent to which hMSCs infected with Delta 24-RGD are capable of localizing throughout human gliomas after systemic injection and are capable of eradicating the tumor by viral oncolysis. In summary, we have successfully translated Delta-24-RGD from the bench to the clinic, and we now propose further clinical and translational studies that will provide the fundamental information necessary for advancing this biological approach as a new and efficacious therapeutic tool.

332 Development of Small Molecule Inhibitors of Leukemogenic CBF Fusion Proteins

Jolanta Grembecka[1], Anuradha Illendula[1], Lauren Dixon[1], Michael Douvas[2], Liya Roudaia[3], Tomasz Cierpicki[1], Yunpeng Zhou[1], Gwen Baber[1], Yan Gao[1], Lucio H. Castilla[4], Nancy A. Speck[5], **John H. Bushweller**[1]

Departments of [1]Molecular Physiology and Biological Physics, and [2]Medicine, University of Virginia Health System; [3]Department of Biochemistry, Dartmouth Medical School; [4]Program in Gene Function and Expression, University of Massachusetts Medical School; [5]Department of Cell and Developmental Biology, University of Pennsylvania Medical School

The genes encoding RUNX1 (AML1) and CBFß are frequent targets of chromosomal translocations leading to the development of leukemia. RUNX1 is the target of the t(8;21) and t(12;21), resulting in expression of AML1-ETO and TEL-AML1 fusions, respectively. CBFB is disrupted by the inv(16), resulting in the expression of the CBFß-SMMHC fusion. In all cases, the fusion proteins act as dominant repressors of CBF function, resulting in substantial alteration of the gene expression program of multiple genes required for normal hematopoiesis and, in cooperation with secondary mutations, leading to the development of leukemia. Most CBF leukemia patients treated with current therapies who achieve hematological and cytogenetic long-term remission retain fusion protein transcripts in their bone marrow that are produced from either leukemic or preleukemic cells that were not eradicated. Thus, although ~90% of patients achieve complete remission, 40–50% of these patients relapse within 5 years due to the resurgence of residual fusion protein expressing cells. We hypothesize that direct therapeutic targeting of the fusion proteins may reduce the rate of relapse and improve the long-term survival of these patients.

To that end, we have validated the interaction between CBFß and the Runt domain in these fusions as an appropriate target for inhibitor development. We have developed assays for this protein-protein interaction to identify small molecule inhibitors. Screening of fragment libraries, virtual screening, and high-throughput screening (HTS) have been employed to identify initial hits. These have been optimized using medicinal chemistry approaches to generate more potent inhibitors. These have been tested for effects on leukemia cell lines and characterized for their pharmacokinetic properties. Efforts to test these compounds in mice are underway. We have also explored novel approaches to achieve selective inhibition of the fusion proteins with minimal effects on the wild-type alleles. Recent results in these areas will be described.

333 Small Molecular Inhibitors of RNA Polymerase II C-Terminal Domain (CTD) With Synthetic Lethality for Oncogenic K-Ras

Wei Guo, Shuhong Wu, Xiaoli Wei, Li Wang, Jinsong Liu, **Bingliang Fang**

The University of Texas M. D. Anderson Cancer Center

K-Ras mutations are frequently found in tumors, especially adenocarcinomas of the pancreas, colon, and lung, and are known to be associated with resistance to chemotherapy and radiotherapy, leading to a poor prognosis. Thus far, there is no effective therapeutic agent available in clinics for K-Ras mutant cancers. Using human ovarian epithelial cells with and without K-Ras transformation, we performed a synthetic lethality screening on a chemical library and identified a small molecule (designated oncrasin-1) that induces synthetic lethality for oncogenic K-Ras. Oncrasin-1 effectively induces apoptosis in various cancer cells. The apoptosis induction correlated with aggregation of protein kinase C iota (PKCι) and splicing factors into mega-splicesomes. Molecular characterization revealed that oncrasin-1 inhibits the phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II. Interestingly, mutations compromising the function of the CTD were reported by others to be synthetically lethal with elevated levels of Ras activity in yeast, suggesting that the CTD may serve as a target for cancer treatment. We have submitted 4 (oncrasin-27, -60, -72, and -73) to the Developmental Therapeutics Program at the National Cancer Institute (NCI) for testing in NCI-60 cancer cell lines. The results showed that these compounds have similar anticancer spectra and are highly active against several cell lines derived from various organs. Moreover, a COMPARE analysis of oncrasin-60 by NCI showed that it lies outside the category of adequately studied classes of antitumor agents, suggesting that oncrasin compounds could be a new class of anticancer agents. More recently, in vivo activity test performed by the Rapid Access to Intervention Development (RAID) Program at NCI showed that treatment with oncrasin-72 caused complete tumor regression in subcutaneous tumors derived from renal cancer cell line A498, without obvious toxicity. Treatment with oncrasin-60 produced tumor regression or stabilization at high and intermediate dose levels, although some weight loss occurred in animals treated with high doses of oncrasin-60. These results demonstrated the in vivo activity of both compounds and suggested possibility of translating them to clinical trials. Thus, oncrasin compounds may represent a novel class of anticancer agents that target to CTD of RNA polymerase II.

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334 Defining the Anti-Tumor Efficacy of Apo2L/TRAIL Against Patient Pancreatic Tumor Xenografts in SCID Mice

Bonnie L. Hylander[1], Ping Liang[2], Rama Dey-Rao[1], John Gibbs[3], Rose Pitoniak[1], Inna Sirota[1], Chen-Ting Lee[1], Elizabeth A. Repasky[1]

[1]Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY; [2]Department of Biological Sciences, Brock University, St. Catharines, Ontario, Canada; [3]Department of Surgery, Roswell Park Cancer Institute, Buffalo, NY

Tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) is a member of the TNF family with promising anti-tumor therapeutic potential and is in clinical trials. The efficacy of Apo2L/TRAIL has been demonstrated in vitro and in vivo largely based on work done with long-established cell lines. We have developed a highly relevant preclinical patient pancreatic tumor/SCID mouse xenograft model to bridge the gap between cell line xenografts and the clinic and to facilitate our understanding of the responsiveness of actual patient tumors to this therapy. Patient tumors grown in SCID mice recapitulate the phenotype of the original tumor and display heterogeneity between tumors, which is seen in the clinic.

Using this model, we have characterized the Apo2L/TRAIL sensitivity of a panel of 16 patient pancreatic tumors. These tumors display a full range of sensitivities to Apo2L/TRAIL, from actual regression (tumor growth inhibition, TGI, of 108%) to complete resistance (TGI of 0%). Importantly, in these patient xenografts, combination therapy with Apo2L/TRAIL and suboptimal doses of chemotherapy has been shown to enhance the antitumor effect against sensitive tumors and, in some cases, to overcome resistance.

We are currently investigating different schedules of administration in order to optimize the anti-tumor efficacy of combination therapy. Three different Apo2L/TRAIL (500μg per dose) schedules are being investigated, and the responses of Apo2L/TRAIL sensitive and resistant pancreatic (and colon tumors) are being compared. We have demonstrated that the least intensive schedule suppresses sensitive tumors, while the response of the resistant tumors appears to be dose-dependent. The results of these experiments will help define the best schedules to be used clinically in order to combine Apo2L/TRAIL with suboptimal doses of chemotherapy in order to control tumor growth while minimizing chemo-induced toxicity.

We are investigating this panel of tumors to identify mechanisms of resistance and approaches to sensitize tumors to Apo2L/TRAIL. Furthermore, we are analyzing sensitive and resistant tumors to identify a gene expression signature that could be used to predict individual patients whose tumors would be expected to be sensitive to Apo2L/TRAIL.

335 Drug Development in Leukemia: Bench to Bedside and Back Again

Judith E. Karp, Michelle Rudek, Mark Levis, Keith Pratz, Linda Resar, B. Douglas Smith, Michael Carducci

Johns Hopkins Kimmel Cancer Center, Baltimore, MD

Leukemia presents a fertile testing ground for drug development. The ready availability of tumor tissue facilitates conduct of longitudinal in vitro and in vivo studies prior to and throughout investigational therapies. Through support of our U01 grant, NCI CA70095, we conduct serial clinical-laboratory trials in poor-risk leukemias, using the initial Phase I trials as springboards for subsequent application and development, based on in vivo laboratory correlative data. In this context, we present studies of four novel agents in adults with acute leukemias at diverse stages of disease: Flavopiridol (F), Sorafenib (S), Tipifarnib (T), and ABT-888. **Flavopiridol:** Based on in vitro data demonstrating that F induces apoptosis in primary human leukemia blasts with recruitment of remaining blasts into S phase, we designed a timed sequential therapy (TST) of F followed by ara-C and mitoxantrone (FLAM). This regimen is active in adults with newly diagnosed, poor risk acute myelogenous leukemias (AMLs), with detection of antileukemic activity in FLT-3 mutant AML. Comparison of "traditional" 1-hour bolus F administration and the Byrd-Blum "hybrid" (bolus-infusion) administration is the subject of a multi-institutional randomized Phase II b trial of FLAM in newly diagnosed, poor-risk AML patients, where pharmacokinetic (PK) and pharmacodynamic (PD) parameters as well as clinical efficacy and toxicity are being compared. **Sorafenib:** In the context of a Phase I trial of S in adults with refractory acute leukemias, detailed PK/PD studies demonstrated that S is a potent inhibitor of mutant FLT-3 activity, with S-N-oxide being the active metabolite and inhibition being durable (at least 7 days post-last dose) based on the novel Plasma Inhibitory Assay (PIA). These PK/PD findings suggest the possibility of intermittent dosing and, for FLT-3 mutations, using low doses in sequence with intensive chemotherapy in FLT3-mutant AML. **Tipifarnib**: Through stepwise clinical-laboratory trials, we have developed T plus etoposide as a clinical therapy for elderly adults with newly diagnosed AML. In this trial, we substantiated in a preliminary fashion a 2-gene signature (initially defined in the context of our Phase II single agent T trial) that predicts for response to tipifarnib. Next steps are to validate and then use the signature prospectively to select appropriate candidates for T therapy. **ABT-888:** PK/PD studies in our Phase I trial demonstrate that ABT-888 accumulates in the marrow compartment as well as in marrow blast cells, as detected by a rapid analytical method based on LC/MS/MS with electrospray positive ionization after a single protein precipitation with acetronitrile.

336 Loss of MicroRNA-122 Promotes Hepatocarcinogenesis: Therapeutic Potential of miR-122 Mimetic Against Liver Cancer

Huban Kutay, Shu-hao Hsu, Bo Wang, Bo Yu, Shoumei Bai, Stefan Costinean, Robert Lee, L. James Lee, Samson T. Jacob, **Kalpana Ghoshal**

Department of Molecular and Cellular Biochemistry, Colleage of Pharmacy, Comprehensive Cancer Center, College of Medicine; Department of Chemical Engineering, The Ohio State University, Columbus, OH

MicroRNAs (miRNAs) are short noncoding RNAs that primarily regulate gene expression at the post-transcriptional level. Numerous biological processes are regulated by miRNAs. The aberrations in microRNA expression lead to different disease states including cancer. miR-122, which constitutes ~70% of the total miRNA in the liver, has been implicated in maintaining differentiation state of the hepatocytes. We were the first to report that the expression of miR-122 is downregulated in both human and rodent HCC (Kutay et al., J. Cellular Biochem. 2006). Suppression of miR-122 in humans correlated with poor prognosis and metastasis of liver cancer. Recently, we showed that miR-122 functions as a tumor suppressor by inhibiting growth, clongenic survival, migration and invasion of HCC cells, and tumor growth in nude mice. Anti-tumorigenic function of miR-122 is mediated through suppression of its targets such as Igf1R, ADAM-10, and SRF. To understand the function of miR-122 in the liver, we have generated conditional knockout (KO) mice (supported by an R21 grant). These mice could potentially be valuable models for different liver diseases. These mice spontaneously develop hepatitis and hepatic dysplasia with age. Since inflammation plays a major role in cancer including hepatocarcinogenesis, it is likely that miR-122KO mice will be markedly more susceptible to liver disease that ultimately leads to HCC. Indeed, our preliminary studies demonstrated facilitation of hepatitis after feeding choline-deficient and amino acid-defined diet that promotes nonalcoholic steatohepatitis (NASH). In addition, these knockout mice are more prone to hepatcarcinogenesis when exposed to diethylnitrosamine, a potent liver carcinogen. Currently, we are testing different nanoparticles for targeted delivery of miR-122 mimetic in the hepatocytes of miR-122KO mice.

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337 Discovery and Development of Novel Ubiquitin Isopeptidase Inhibitors

Craig A. Leach[1], K.G. Suresh Kumar[1], Seth J. Goldenberg[1], Jeffrey L. McDermott[1], Matthew P. Kodrasov[1], Dharminder Chauhan[2], Ze Tian[2], Kenneth C. Anderson[2], Michael R. Mattern[1], Benjamin Nicholson[1].

[1]Progenra, Inc, Malvern, PA; [2]Dana-Farber Cancer Institute, Boston, MA

The ubiquitin-proteasome is a complex system consisting of enzymes that conjugate and deconjugate ubiquitin to/from target proteins upstream of the 26S proteasome. In particular, ubiquitin deconjugation is performed by families of proteases known collectively as deubiquitylating enzymes (DUBs). The therapeutic validation of the ubiquitin-proteasome system was provided by the marketing approval of Velcade® for the treatment of multiple myeloma (MM).

Mutations in pVHL are observed in 100% of patients with the autosomal dominant von Hippel-Lindau disease (VHL) and predispose individuals to a variety of tumors including clear cell carcinomas of the kidneys. In addition, mutations in pVHL are observed in 50–80% of patients with the more common sporadic renal clear cell carcinoma. Significantly, two related DUBs—USP33 (VDU1) and USP20 (VDU2)—are substrates of the pVHL E3 ligase complex and hence are implicated in carcinogenesis. The tight regulation of USP20 and USP33 protein levels by the tumor suppressor pVHL support the hypothesis that USP20 and USP33 proteins play a role in carcinogenesis. Many other DUBs play critical roles in cellular function, including USP7. USP7 regulates the degradation of many proteins including the oncogene hDM2 and the DNA repair protein claspin.

In our search for novel chemotherapeutic agents to treat cancer, we investigated the therapeutic potential of inhibiting several members of the DUB family including USP20, USP33, and USP7. Screening of small molecule compound libraries identified several inhibitors including P005091, a selective USP7 inhibitor. Cell-based pharmacodynamic experiments revealed that P005091 enhanced the degradation of the USP7 substrates Hdm2 and claspin in tumor cells. In addition, P005091 inhibits the growth of ex vivo human tumor cells but has no effect on normal cells. Subsequent medicinal chemistry efforts identified novel, potent, and selective inhibitors of USP7 that are being further optimized for progression into clinical studies.

338 A Novel E2f Inhibitor, Hlm006474, Downregulates Expression of Mitotic Genes and Synergizes With Other Chemotherapeutic Compounds

Yihong Ma, Courtney A. Kurtyka, Haikuo Bian, Steve Enkemann, Christopher Cubit, **W. Douglas Cress**

Molecular Oncology Program, H. Lee Moffitt Cancer Center

HLM006474 was identified using a computer-based virtual screen and the known crystal structure of the DNA bound E2F4/DP2 heterodimer. Treatment of multiple lung cancer cell lines with HLM006474 resulted in the loss of intracellular E2F4 activity. HLM006474 dramatically affects expression of a gene expression signature characteristic of highly mitotic lung cancers. The effects of HLM006474 treatment on different cell lines varied but included a reduction in cell proliferation and an increase in apoptosis. All NSCLC lines tested so far are sensitive to HLM006474, with a typical IC50 of approximately 30 μM. HLM006474 synergizes with a novel cdk inhibitor, taxol and dasatinib but not with cisplatin and gemcitabine. Together, these results suggest that interference with E2F activity using small molecules may have clinical application in lung cancer therapy.

339 2'-Deoxy-4'-C-Ethynyl-2-Fluoroadenosine (EFdA), a Novel 4'-C-Ethynyl Nucleoside Analog Highly Potent Against Multidrug-Resistant HIV-1 Variants

Kenji Maeda[1], Michael A. Parniak[2], Michael Murphey-Corb[2], Hirotomo Nakata[1], and Hiroaki Mitsuya[1]

[1]National Cancer Institute; [2]University of Pittsburgh School of Medicine, Pittsburgh

2'-Deoxy-4'-C-ethynyl-2-fluoroadenosine (EFdA) is potently active against a wide spectrum of HIV isolates including wild-type and drug-resistant HIV strains in vitro with IC50 values of ~1 nM. When human CEM cells were incubated with 200 nM 3H-EFdA or 3H-3'-azido-2',3'-dideoxythymidine (AZT) and nucleosides/nucleotides within the cells were quantified, amounts of intracellular EFdA-monophosphates (MP), -diphosphates (DP), and -triphosphates (TP) increased proportionately with increased EFdA concentrations. Intracellular T1/2 of EFdATP, an active form of EFdA, was >12 hr in CEM cells, which was significantly greater than T1/2 of AZTTP (~3 h), suggesting that once or twice daily dosing schedule of EFdA could be possible. The IC50 value of EFdATP to inhibit dATP (0.3 μM) incorporation mediated by DNA polymerase γ was 10 μM, which was significantly higher than that of ddATP (IC50 value: 0.2 μM). We also found that EFdA exerts minimal inhibition to the activity of human DNA polymerases α and ß.

EFdA was also potent against simian immunodeficiency virus (SIV)(IC50=50 pM), and we examined the activity of EFdA in SIV-infected rhesus macaques. Two macaques exhibiting end-stage simian immunodeficiency syndrome (SAIDS) had been previously treated with 9-R-2-phosphonomethoxypropyl-adenine (PMPA) for 10 months but were without drug exposure for approximately 2 years prior to the initiation of EFdA treatment. The macaques, which had reached end-stage SAIDS as defined by chronic diarrhea, loss of greater than 20% of total body weight, and persistent high plasma virus loads, were treated with once daily dosing (QD) of EFdA (2 mg/kg, subcutaneous injection). Within 1 week of EFdA treatment, both animals showed a 3–4 log decrease in plasma virus load; this declined to undetectable within 1–2 months. Chronic diarrhea resolved in both animals within one month, and both showed steady gains in body weight for the duration of EFdA treatment. Liver enzyme panels remained normal for the entire course of EFdA treatment.

The data of antiviral and pharmacological properties of EFdA show that the compound is potent against a wide spectrum of HIV-1 variants with minimum cytotoxicity. The pilot study in SIV-infected macaques shows that QD administration of EFdA is highly effective in reducing plasma virus load and in resolving SAIDS symptoms. The drug appeared safe as no hepatoxicity was seen over the 6-month course of treatment. The activity/safety profiles of EFdA and the pilot study results in macaques suggest that more extensive non-human primate evaluations of EFdA antiretroviral efficacy are warranted.

340 RNAi Analysis and Screening for the Study of Cancer Gene Function and Anti-Cancer Therapeutics

Scott E. Martin[1], Philip Lorenzi[2], Jenny Llamas[1], Amanda B. Hummon[3], Jordi Camps[3], Marian Grade[3], Yong-Wei Zhang[4], Lyndsay Murrow[5], Sireesha Garimella[5], Kristen Gehlhaus[1] Tamara Jones[1], Konrad Huppi[1], Mark Mackiewicz[1]. John Weinstein[2], Stanley Lipkowitz[5], Thomas Ried[3], Yves Pommier[4], **Natasha J. Caplen**[1]

[1]Gene Silencing Section, Genetics Branch, Center for Cancer Research, National Cancer Institute; [2]Department of Bioinformatics & Computational Biology, M. D. Anderson Cancer Center, TX; [3]Cancer Genomics Section, Genetics Branch, [4]Laboratory of Molecular Pharmacology, and [5]Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute

The identification of the RNAi mechanism in mammalian cells has had a profound impact on our understanding of the post-transcriptional regulation of gene expression. Further, the discovery of RNAi has provided the scientific community with a powerful means for conducting loss of function (LOF) analysis in a gene-specific manner. To probe the function of cancer-associated genes, we are using a combination of large scale RNAi screening, gene specific RNAi analysis, whole transcriptome analysis, and downstream functional analysis. Using this approach, we have identified novel molecular targets for the treatment of triple-negative/basal-type breast cancer, ovarian cancer, and colorectal cancer. RNAi-based technologies also have the potential to enhance many aspects of the anti-cancer drug development process. For example, we are using RNAi analysis and screening to probe the impact of specific genes on drug-activity, including study of a relationship between asparaginase synthetase (ASNS) gene expression and sensitivity to the anti-leukemia agent L-Asparaginase. We are also using a chemosensitization (synthetic lethal) approach that combines the decrease of a protein through RNAi with administration of a small molecule or biologic to identify new proteins that directly or indirectly modulate the pharmacology of anti-cancer therapeutics. This RNAi screening approach has the potential to enhance the clinical application of an established or investigational drug by (1) identifying synergistic molecular targets that exploit complementary vulnerabilities within a cancer cell, (2) enabling the use of lower concentrations of a drug that exhibits dose-dependent non-specific toxicities, (3) overcoming drug resistance, and (4) broadening the clinical application of a drug to other cancer types. This approach can also be used to identify antagonists of drug activity that may be relevant to the response of patients to drugs. To date, our chemosensitization RNAi screens have led us to investigate the role of the ribonucleotide reductase complex in the cellular response to the topoisomerase 1 inhibitor camptothecin (CPT). We have also identified MAP3K7 (TAK1) as a potent agonist of CPT and its clinical derivatives. These studies illustrate the importance of RNAi analysis and screening in translational research with a goal of directly impacting therapeutic approaches to cancer.

341 Manipulation of Sphingolipid Metabolism: A Strategy for Improving Chemotherapy

James S. Norris[1], Besim Ogretmen[2], Lina M. Obeid[3], Yusuf A. Hannun[2], Alicja Bielawska[2], Xiang Liu[1]

[1]Department of Microbiology and Immunology, [2]Department of Biochemistry and Molecular Biology, [3]General Internal Medicine/Geriatrics, Medical University of South Carolina

The mechanism in cancer cells causing therapeutic resistance has been extensively studied, but attempts to overcome this problem have been only marginally successful. While addressing this, we and others linked aberrant sphingolipid metabolism to chemotherapy-related drug resistance. It is well known that most chemotherapy drugs act in part by modulating, in cancer cells, the sphingolipid signaling pathways that culminate in programmed cell death or paradoxically survival in some cases. Ceramide, the basic structural unit of sphingolipids, plays a central role in cell death signaling. Recent evidence from our group and others suggests that aberrant ceramide metabolism is critically involved in cancer pathogenesis and chemo-resistance and suggests that manipulation of sphingolipid metabolism may be a way to circumvent the insensitivity of cancer cells to chemotherapy. Three strategies to address this are underway in our group and seem particularly promising in preclinical cancer models. One is to drive sphingolipid metabolism towards the production of the proapoptotic lipid ceramide, which leads to cell death. Conversely ceramide deacylation leads to the production of sphingosine, the substrate for sphingosine kinase that catalyzes formation of sphingosine-1-phosphate, a potent anti-apoptotic angiogenic lipid. To prevent deacylation, we have developed lysosomal targeted acid ceramidase inhibitors that permit induction of ceramide following chemotherapy and overcome this form of resistance. The second approach was development of organelle-targeted ceramide analogs that increase mitochondrial ceramide and subsequent cell death. Both sets of drugs represent potential new classes of agents that augment traditional chemotherapy approaches. Not discussed today is a third approach that has focused on development of inhibitors of sphingosine kinase. We have published on these subjects and have demonstrated the success of the first two groups of drugs in preclinical models. Today, I wish to focus attention on one of our leading drug candidates, L-t-Pyr-Cer (SPG103), a water soluble-mitochondrial targeted molecule that raises the level of ceramide in the mitochondria. This drug is shown to accumulate specifically in mitochondria, and in vivo it appears to concentrate preferentially in tumor tissue. It induces apoptosis in our model systems. Our preclinical data suggest that combination treatment of SPG103 and Gemcitabine in several cancer models provides an overall survival advantage.

342 Discovery of Anticancer Agents of Diverse Natural Origin

Jimmy Orjala[1], George Chlipala[1], Aleksej Krunic[1], Steven M. Swanson[1], John T. Hunt[2], Stuart Emanuel[2], Nicholas H. Oberlies[3,4], Cedric J. Pearce[5], A. Douglas Kinghorn[6]

[1]College of Pharmacy, The University of Illinois at Chicago; [2]Bristol-Myers Squibb, Pharmaceutical Research Institute, Princeton, NJ; [3]Research Triangle Institute, Research Triangle Park, NC; [4]University of North Carolina-Greensboro; [5]Mycosynthetix, Inc., Hillsborough, NC; [6]College of Pharmacy, The Ohio State University, Columbus, OH

Natural products and their derivatives continue to be an important source for novel anticancer drugs. As part of a multi-disciplinary collaborative project directed towards the discovery of novel anticancer agents entitled "Discovery of Anticancer Agents of Diverse Natural Origin" (P01 CA125066), we are investigating the secondary metabolites of aquatic cyanobacteria, tropical plants, and filamentous fungi. Extracts are evaluated in variety of mechanistic and cell-based assays germane to cancer. Pure compounds are isolated from the most potent and selective extracts using activity-guided fractionation protocols followed by lead prioritization for potential further development. The presentation will focus on our recent discoveries of novel proteasome inhibitors from cultured cyanobacteria. These examples will serve as an illustration to outline the multi-disciplinary collaborative nature of our project.

343 Phase I Clinical Trial of Fludarabine Phosphate in Conjunction With E. Coli Purine Nucleoside Phosphorylase

William B. Parker[1], Eric J. Sorscher[2]

[1]Southern Research Institute, Birmingham, AL; [2]University of Alabama at Birmingham

We have developed a gene therapy strategy for the treatment of solid tumors based on the selective activation of fludarabine in tumor cells by E. coli purine nucleoside phosphorylase (PNP). Fludarabine phosphate (an FDA-approved drug currently used to treat chronic lymphocytic leukemia but not active against solid tumors) is rapidly converted by plasma phosphatases to fludarabine, which is a substrate for E. coli PNP, but not human PNP. E. coli PNP catalyses the conversion of fludarabine to 2-fluoroadenine, which has demonstrated potent anti-cancer activity against numerous solid tumor cell lines in vitro. Selective expression of E. coli PNP in tumor cells has been shown to dramatically increase the anti-cancer activity of fludarabine phosphate in human tumor xenografts in mice without increasing the toxicity of the compound. Fluoroadenine readily diffuses to neighboring tumor cells that do not express E. coli PNP, and the strategy confers excellent in vivo bystander activity against human tumor xenografts in vivo. In addition, fluoroadenine is active against non-replicating tumor cells due to its conversion to 2-fluoro-ATP and subsequent inhibition of RNA and protein syntheses, a mechanism of action different from all currently used anti-cancer agents. The E. coli PNP gene has been inserted into a first-generation, replication-defective adenoviral vector (E1 and E3 deleted), and injection of this vector into subcutaneous solid tumors followed by systemic treatment with fludarabine phosphate results in good antitumor activity. PNP Therapeutics, Inc., has licensed the anti-cancer strategy and has evaluated safety in healthy rats and tumor-bearing mice in preparation for a phase I clinical trial to evaluate the safety (and efficacy) in patients with end-stage head and neck tumors. In a GLP-compliant toxicology study in rats where fluoroadenine was administered intravenously daily for 7 consecutive days, the MTD of fluoroadenine was 18 mg/m2, and the toxicities observed were typical of those seen with nucleoside analogs. In a disease toxicology model, mice were administered adenoviral vector intratumorally with fludarabine phosphate intravenously in a manner similar to that proposed for the phase I clinical trial. In this study, no acute toxicities were observed, and there was only mild liver toxicity seen at a dose of vector 10-fold greater than the clinical dose. The first patients are expected to be enrolled in the trial in the first quarter of 2010.

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344 Comprehensive Modeling of Cancer Pathways

Richard G. Posner[1], Joshua Colvin[1] Mathew Creamer[2], Daniel D. Von Hoff[1]

[1]Clinical Translational Research Division, Translational Genomics Research Institute, Phoenix, AZ; [2]Department of Biological Sciences, Northern Arizona University, Flagstaff

For a given cancer phenotype, individual patients generally express a subset of a much larger set of genetic mutations found among the population of patients with this type of cancer. These mutations typically affect proteins of signal-transduction systems. It is clear that accumulation of molecular changes of signaling proteins and changes in the expression levels of these proteins can be oncogenic, but it is not clear what the controlling principles are that govern the transformation to malignancy.

Because signaling networks are so highly interconnected and dynamic, it is difficult to use intuition or qualitative methods to predict how a cell will respond to perturbations, such as molecularly-targeted drug therapy. When intuition fails, a predictive understanding of a complex dynamic system can often be obtained through mathematical modeling. We believe that the development of models for cancer pathways is essential for understanding the design principles of these pathways and the underlying reasons for the dysfunctional behaviors of cancer cells. While such models have the potential to guide rational drug discovery, clinical practice, and diagnosis, they depend on multisite protein modifications that can give rise to combinatorial complexity by which an enormous number of potential molecular configurations can be generated. This combinatorial potential of proteins and protein-protein interactions has posed a barrier to the development of mechanistic comprehensive models as even a relatively simple model can generate trillions of unique chemical species. The size of such networks overloads conventional modeling methods.

We have recently developed a novel computational method for specifying and calculating the dynamics of large-scale signaling networks. With our software implementation of this new method (Colvin et al., 2009), it is now possible to contemplate building and analyzing models that include a significant fraction of the protein interactions that comprise a cancer pathway, with incorporation of the site-specific details of the interactions. We believe modeling at this level of detail will be important for understanding cancer pathways because these pathways are affected by mutations of protein sites that subtly alter protein function. We are applying this method to the construction of mechanistic models of signaling via TGFß and ErbB receptors and the cell cycle. We demonstrate this approach to formulate precise hypotheses on the specific changes anticipated at signaling nodes in these networks that occur upon addition of siRNA or targeted drug therapy to our various pancreatic cancer cell lines.

345 Enhancing TRAIL Onduced Apoptosis by XIAP

Vinay K. Puduvalli, Yuanfang Liu, Frederick Lang, Jihong Xu

The University of Texas M. D. Anderson Cancer Center, Houston

Multiforme enhances the viability of malignant cells by activating survival pathways and inhibiting pro-apoptotic factors. X-linked inhibitor of Apoptosis (XIAP) is a downstream target of Akt that can potently inhibit caspase 3 and caspase 9 and abrogate both death receptor and mitochondrial pathways of apoptosis. TNF-related apoptosis inducing ligand (TRAIL), by activating the death receptor pathway, potently induces apoptosis in gliomas, an effect which can be inhibited by XIAP. We have demonstrated that Ad-sTRAIL, an adenoviral vector that expressed soluble TRAIL, can induce apoptosis in gliomas and increase survival in an intracranial glioma xenograft model. We hypothesized that inhibition of XIAP could enhance TRAIL-induced apotosis and improve the survival advantage provided by Ad-sTRAIL. To test this hypothesis, U251HF and SNB19 glioma cells were treated with Ad-XAF1 (adenoviral construct expressing the endogenous XIAP inhibitor, XAF1) or with Compound #12 (a chemical inhibitor of XIAP) and relevant controls and exposed to soluble TRAIL (sTRAIL). Inhibition of XIAP significantly enhanced sTRAIL induced apoptosis in vitro. Using a novel ex vivo organotypic human glioma slice model, we observed a similar increase in the degree of cytotoxicity with the combination of AdsTRAIL and AdXAF1. Next, we examined the efficacy of this combination in a mouse intracranial glioma xenograft model with tumors derived from implanted U251HF-Luc cells that constitutively express luciferase and can be monitored by bioluminescent imaging. Intratumoral injection of AdEGFP, AdXAF1, AdsTRAIL, or AdXAF1+AdsTRAIL alone along with appropriate controls was performed biweekly for 3 weeks. Change in tumor size was measured as a function of quantitative bioluminescent imaging and the survival recorded. Tumors treated with the combination of AdXAF1 and AdsTRAIL showed a progressive decrease in bioluminescence compared with other treatment conditions. Correspondingly, a statistically significant increase in survival was seen in AdXAF1+AdsTRAIL-treated animals compared with either agent alone or controls. Our results show that the efficacy of AdsTRAIL was significantly improved by inhibition of XIAP and suggests a potential locoregional strategy using these agents for treatment of malignant gliomas.

346 Proteasome Inhibitor Therapy in Multiple Myeloma

Paul Richardson, Teru Hideshima, Dharminder Chauhan, Nikhil Munshi, **Kenneth C. Anderson**

Dana-Farber Cancer Institute, Boston, MA

Bortezomib (BZ) targets the MM cell in the bone marrow microenvironment to overcome conventional drug resistance in laboratory and animal models; it was effective to treat advanced MM and has rapidly moved into the management of newly diagnosed MM. However, not all patients respond to Bortezomib, and those that do ultimately develop resistance. Two strategies, development of next-generation proteasome inhibitors and rationally designed BZ combination therapies, are under evaluation to overcome clinical BZ resistance. First, several next-generation proteasome inhibitors overcome BZ resistance in preclinical models. Carlfizomib more potently inhibits the chymotyrptic activity: responses have been observed in relapsed and refractory MM, without neuropathy. Responses to NPI-0052, which targets chymotryptic, tryptic-like, and caspase-like activities, have also been observed. Second, BZ can be used in combination treatments to overcome resistance. BZ inhibits DNA damage repair, and a phase III trial demonstrated significantly increased extent and frequency of response as well as duration of response and overall survival in patients treated with BZ with pegylated doxorubicin versus BZ alone, leading to its FDA and EMEA approval. BZ and lenalidomide trigger primarily caspase 9 and 8 mediated apoptosis, respectively; the combination triggers dual apoptotic signaling and synergistic death in vitro. This combination achieves 58% response in patients with relapsed refractory MM and 100% responses with 71% very good partial response in newly diagnosed disease. BZ has been combined with heat shock protein (hsp) 90 inhibitor tanespimycin to block its induction by BZ and mediates synergistic preclinical cytoxicity; addition of tanespimycin can sensitize or overcome clinical resistance to BZ, and a phase III trial is comparing BZ versus BZ and tanespimycin in relapsed MM. Since BZ activates Akt in vitro, we have combined the Akt inhibitor perifosine with BZ to trigger synergistic MM cell death. BZ with perifosine achieves responses in patients with MM refractory to BZ, and a phase III clinical trial evaluating BZ and perifosine versus BZ in relapsed MM is ongoing. Finally, blockade of aggresomal and proteasomal degradation of proteins by histone deacetylase inhibitors and BZ, respectively, is synergistic in preclinical studies; the histone deacetylase inhibitor vorinostat with BZ shows benefit in the majority of patients with relapsed refractory MM. Future translational research will define scientifically-based combination therapies to achieve high frequency and durable responses in the majority of patients with MM.

347 Identification and Validation of NOL5A and RPS2 as Potential Therapeutic Targets in Colorectal Cancer Using a Functional Genomics Approach

Thomas Ried, Amanda Hummon, Jordi Camps, Georg Emons, Michael J. Difilippantonio, Natasha Caplen, Marian Grade

National Cancer Institute

Despite major improvements in elucidating the genetic changes underlying the initiation and progression of colorectal cancer (CRC), there remains a clinical need to implement novel targeted therapeutic strategies. Proteins that are highly overexpressed in tumor cells have the potential to be selective therapeutic targets. We have previously defined CRC-specific gene expression profiles that include many overexpressed protein-encoding genes. Using cell-based model systems that recapitulate the genomic and gene expression changes we have previously observed in primary CRC, we applied a functional genomics strategy to identify such anti-CRC targets. To identify potential therapeutic targets, we assessed the functional and molecular consequences of RNAi-mediated silencing of candidate genes chosen from within these profiles. RNAi against five of these genes, HMGA1, RRM2, TACSTD2, RPS2 and NOL5A, resulted in a marked viability reduction of CRC cell lines. Unique RNAi gene expression signatures generated for each of these genes showed enrichment for specific cellular pathways, and correlation of these signatures with expression profiles from independent clinical samples suggests that RPS2 and NOL5A are promising new therapeutic targets in CRC.

348 Small Molecule Discovery by High Throughput Flow Cytometry

Larry A. Sklar

The University of New Mexico Cancer Center

The University of New Mexico Cancer Center (P30CA118100) in conjunction with the NIH Roadmap Molecular Libraries Initiative (http://mli.nih.gov/mli/) has developed a small molecule screening and probe development resource based on high throughput flow cytometry. The resource (U54MH084690, http://screening.health.unm.edu/) collaborates with investigative teams at National Cancer Institute-designated cancer centers and elsewhere in small molecule discovery projects. Cancer relevant targets have included nuclear estrogen receptors and GPR30, ABC transporters, Bcl-2 family proteins, FPR family receptors, RGS family proteins, small GTPases, metalloproteases, and MEK family protein binding domains. These screens have generated probe compounds and several million screening data points, which are published at the PubChem website (http://pubchem.ncbi.nlm.nih.gov/). Biological content is increased with mixtures of targets that are color-coded as suspension arrays. The GTPase family screens, for example, reveal both small molecule activators and inhibitors with intracellular activities. We are exploring yeast multiplex model systems for TOR pathway analysis, cell cycle regulation, transporters, and yeast two hybrid protein-protein interactions. The HyperCyt flow cytometry platform and its continuing development have the potential of fulfilling a unique niche in small molecule identification for cell and molecular assays in suspension, especially in complex cell suspensions for primary cells, hematopoietic stem cells, and leukemia.

349 AS1411, The First Aptamer in Clinical Oncology Trials

Sridharan Soundararajan[1], Li Wang[1], Eleanor Spicer[1], Robert Stuart[1], Fiona McLaughlin[2], **Daniel J. Fernandes**[1]

[1]Hollings Cancer Center, Medical University of South Carolina, Charleston; [2]Antisoma Research Ltd, BioPark Hertfordshire, Welwyn Garden City, United Kingdom

AS1411 is a 26-nucleotide unmodified DNA aptamer that is currently in a Phase II clinical trial for renal cell carcinoma and has recently completed a Phase II trial for the treatment of relapsed or refractory acute myeloid leukemia (AML). This aptamer binds tightly and specifically to the protein nucleolin, which we have shown is highly overexpressed in the plasma membrane and cytoplasm of human AML, CLL, and breast cancer cells compared to the corresponding normal CD33+ myeloid cells, CD19+ B cells, and normal mammary epithelial cells. Plasma membrane nucleolin is a receptor for AS1411, and AS1411 is thought to gain intracellular access when nucleolin is shuttled from the plasma membrane to the cytoplasm and nucleus. In addition, we have shown that nucleolin binds to an ARE-instability element in the 3'UTR of bcl-2 mRNA. This protects bcl-2 mRNA from degradation and allows the tumor cells to upregulate Bcl-2 protein and avoid apoptosis. AS1411, by acting as a molecular decoy, competes with bcl-2 mRNA for binding to nucleolin and thereby induces bcl-2 mRNA instability, Bcl-2 protein downregulation, and apoptosis. This occurs to a much greater extent in tumor cells than in normal cells because normal cells do not overexpress nucleolin in the cytoplasm. Thus, tumor-targeting of AS1411 appears to be based on the selective uptake of AS1411 into tumor cells and its downregulation of bcl-2 mRNA and protein in these cells. Recently, a 70-patient multicenter, randomized Phase II trial of AS1411 + Cytarabine vs. Cytarabine alone in refractory or relapsed AML was completed. The response rate (CR + CRp) was significantly higher in the AS1411 + Cytarabine arm (21%) than the Cytarabine alone arm (5%). The addition of AS1411 to Cytarabine was well tolerated, and the higher response rate may have resulted from Bcl-2 downregulation by AS1411 in the drug-resistant AML cells.

350 Vesicular Stomatitis Virus as a Treatment for Colorectal Cancer

John H. Stewart, IV[1], Scott A. Northrup[1], Maryam Ahmed[2], Doug S. Lyles[2]

Departments of [1]General Surgery, Section on Surgical Oncology and [2]Biochemistry, The Wake Forest University School of Medicine, Winston-Salem, NC

Introduction: Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States. Vesicular stomatitis virus (VSV) is an attractive candidate as an oncolytic virus for the treatment of CRC due to its potent ability to induce apoptosis. Our group has generated a genetically engineered recombinant VSV containing an M protein mutation (rM51R) to enhance its tumor selectivity while preserving its oncolytic effects. As an extension of these findings, we hypothesized that oncolytic VSV can effectively treat CRC cells that are defective in their antiviral responses and therefore serve as an oncolytic therapy for the treatment of metastatic CRC.

Methods: We compared the cytolytic effects of wild-type (rwt) and M51R VSV in RKO, HCT116, and Lovo CRC cell lines via MTS assays. We initially sought to determine whether CRC cells were susceptible to the oncolytic effects of VSV in single and multiple-cycle infection conditions. Viral protein synthesis and the production of viral progeny by CRC cell lines was quantified by S35 methionine labeling and viral plaque assays, respectively. RKO tumor xenografts were established in athymic nude mice and injected with 10*6 pfu of M51R VSV.

Results: We found that CRC cells display differential sensitivity to oncolytic VSV. RKO and HCT116 CRC cells were very sensitive to the oncolytic effects of both rwt and M51R viruses at 48 hours post-infection, while Lovo cells were somewhat resistant to VSV-induced cell death. All CRC cell lines supported the production of viral progeny, with the peak viral production occurring at 36 hours after infection with rwt and M51R VSV. Although all cell lines supported viral protein production, host protein production was significantly reduced at 8 hours post-infection with M51R virus in RKO and HCT116 CRC cell lines while host protein production was not reduced in the resistant Lovo CRC cells. Treatment of RKO xenografts with M51R VSV resulted in stabilization of tumor size relative to untreated controls after 21 days.

Conclusions: These findings suggest that M51R VSV is a good candidate oncolytic virus for the treatment of metastatic colorectal cancer. Our future work will focus on defining the proportion of colorectal cancers that are susceptible to the oncolytic effects of VSV. In addition, we will identify molecular targets that may enhance the therapeutic efficacy of M51R VSV.

351 Arsenic Trioxide Plus Ethacrynic Acid and Its Non-Diuretic Analogue Are Synergistic to Induce Apoptosis in Non-APL Myeloid Leukemia Cells

Rui Wang, Changda Liu, Samuel Waxman, **Yongkui Jing**

Mount Sinai School of Medicine, New York

Arsenic trioxide (As2O3) induces complete remission in acute promyelocytic leukemia (APL) patients. However, other types of myeloid leukemia patients are less responsive to As2O3 treatment. APL t(15;17) NB4 cells are more sensitive to As2O3 apoptosis induction than other types of myeloid leukemia cells such as HL-60 and K562 cells. Previously we reported that As2O3 induces apoptosis in NB4 cells through a reactive oxygen species (ROS)-mediated pathway that is attenuated by the expression of glutathione-s-transferase pi (GSTpi). NB4 cells contain lower levels of GSTpi than other types of myeloid leukemia cells. Therefore, inhibition of GSTpi should enhance As2O3 induction of apoptosis in other types of myeloid leukemia cells. We investigated the combined effect of As2O3 with ethacrynic acid (EA) and a more potent non-diuretic EA analogue ethacrynic acid-butyl ester (EABE), known GSTpi inhibitors, to induce apoptosis in HL-60 and K562 cells. As2O3 at clinical achievable concentrations (1-2 μM) did not induce apoptosis in HL-60 cells nor in K562 cells. EA at a concentration lower than 60 μM did not induce apoptosis in both cell lines either. As2O3 at 2 μM plus EA at 60 μM are synergistic to induce apoptosis in both HL-60 and K562 cells as measured by Annexin V staining, PARP cleavage, and caspase activation. More intriguing, EABE at 1 μM (60 fold lower than EA) exhibited synergistic induction with As2O3 in HL-60 cells. Combined indexes of As2O3 with EA or EABE on apoptosis induction in HL-60 cells calculated using CompuSyn software are less than 1.0, which indicates synergism. Apoptosis induction by the combination treatment of As2O3 with EA correlated with the downregulation of inhibitor of apoptosis proteins (IAPs), survivin and XIAP. Combination treatment of As2O3 with EA in K562 cells increased the activity of c-Jun N-terminal kinase (JNK) as determined by measuring the levels of phosphorylated JNK and phosphorylated c-Jun. Since GSTpi detoxifies As2O3 and inhibits JNK activation, inhibition of GSTpi activity by EA and its analogue resulted in greater ROS production and activation of JNK. The combined effects of As2O3 with EA and its analogue in fresh AML cells and animal models are under investigation.

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352 A Critical Role for CDC25C and PP2A in the Selective Suppression of Deletion 5q MDS and Drug Resistance by Lenalidomide

Sheng Wei, Xianhong Chen, Kathy Rocha, Ling Zhang, Lubomir Sokol, Justine Clark, P.K. Burnette, Alan List

Immunology Program and Malignant Hematology Program, Moffitt Cancer Center and Research Institute, Tampa, FL

Myelodysplastic syndrome (MDS) represents important rare diseases and healthy issues in the expanding aged population due to the lack of understanding, challenging molecular mechanisms, and poor treatment options. Lenalidomide is the first karyotype-selective therapeutic approved for the treatment of MDS owing to high rates of erythroid and cytogenetic response in patients with chromosome 5q deletion [del(5q)]; however, the mechanism and the molecular targets of lenalidomide that account for its selective activity in MDS are not fully understood. Our previous clinical and laboratory investigations show that lenalidomide promotes erythropoiesis in MDS patients that have del(5q). We then further described possible haplodeficient enzymatic targets that are encoded within the CDR that have key roles in cell cycle regulation. We showed that the dual specificity phosphatases, Cdc25C and PP2A-Cα, which are co-regulators of the G2-M checkpoint, are inhibited by lenalidomide. Lenalidomide-inhibited phosphatase activity either directly (Cdc25C) or indirectly (PP2A) with corresponding retention of inhibitory phospho tyrosine residues. Treatment of del(5q) AML cells with lenalidomide induced G2 arrest and apoptosis, whereas no changes were observed in non-del(5q) AML cells. Small interfering RNA (siRNA) suppression of Cdc25C and PP2A-Cα gene expression recapitulated susceptibility to lenalidomide with induction of G2 arrest and apoptosis in both U937 and primary non-del(5q) MDS cells. Furthermore, patients who failed to respond to lenalidomide therapy displayed high levels of Cdc25C and PP2A expression by immunohistological staining when compared with patients who are sensitive to lenalidomide treatment. Importantly, these same patients also had high levels of P53 protein. Double knockdown of Cdc25C and PP2A in U937 cells caused increased P53 accumulation and expression, indicating that P53 expression may be linked to the regulation of apoptosis and cell-cycle arrest in hematopoietic cell in MDS patients. The increased PP2A expression may be responsible for the lenalidomide resistance in patients who fail to respond to this therapy because PP2A interacts with MDM2, a co-regulator and stabilizer of P53, therefore leading to P53 accumulation. These data establish a model proposing that the upregulated expression of Cdc25C and PP2A seen in MDS patients may coordinate with MDM2 and consequently P53, resulting in apoptosis and cell-cycle arrest of hematopoietic cells. This report is the first to describe the mechanism involved in MDS pathogenesis and lenalidomide resistance seen in MDS patients.

353 Phase I Clinical Translation Trial of Oncolytic rVSV Virotherapy for HCC

Savio L.C. Woo

Department of Gene and Cell Medicine, Mount Sinai School of Medicine, New York

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths in the world, accounting for over 1 million cases annually. Median survival of untreated HCC patients is 7.8 months with a 3-year survival rate of 10%. Conditionally replicating viruses targeted to tumors are being developed as a novel class of oncolytic agents for cancer treatment. Vesicular Stomatitis Virus (VSV) is a cytoplasmic RNA virus with inherent specificity for replication in tumor cells due to their attenuated anti-viral responses. The safety profile of our vector is improved over wild-type VSV by deletion of the amino acid at position 51 of the matrix protein, which renders it unable to block cellular mRNA transport, leading to elevated interferon and cytokine expression in virus-infected cells. To enhance the oncolytic property of rVSV(MΔ51), we incorporated the gene for the secreted form of the murine gammaherpesvirus M3, a viral chemokine binding protein that binds to a broad range of chemokines with high affinity. When administered via hepatic artery infusion, rVSV(MD51)-M3 infected and replicated extensively in advanced multi-focal hepatoma lesions pre-established in the livers of syngeneic and immune-competent rats leading to 50% long-term survival. It blocked inflammatory cell migration to the tumor site, which allowed for enhanced intratumoral virus replication leading to increased tumor necrosis. No toxicities were noted based on analyses of serum aminotransferase and proinflammatory cytokine levels or in the liver by histopathologic evaluation. Our preliminary data, pharmlogical/toxicological study designs, and clinical protocol have been discussed extensively with the FDA through a series of pre-IND conference calls and presented to OBA at a public meeting of the RAC. We are performing a series of preclinical pharmalogical and toxicological studies designed in accordance with their suggestions, which include biodistribution in tumor-bearing rats and the assessment of acute and long-term toxicity in normal rats and normal rhesus monkeys. A clinical lot of rVSV(MΔ51)-M3 under cGMP for use in the dose escalation trial in HCC patients has been produced and fully characterized. The successful conduct of the clinical translational research may lead to the development of recombinant VSVs as effective and safe oncolytic agents to treat patients with advanced HCC and other cancers in the future.

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354 Targeting Hypoxic Microenvironment in Acute Myelogenous Leukemia (AML)

Michael Andreeff, Zihong Zeng, Olga Frolova, Sergej Konoplev, Michael Fiegl, Ismael Samudio, Juliana Benito, Yuexi Shi, Rodrigo Jacamo, Hongbo Lu, Carlos Bueso-Ramos, Deborah A. Thomas, Hagop Kantarjian, Marina Konopleva

University of Texas M. D. Anderson Cancer Center

The main therapeutic challenge in acute leukemias is the development of strategies overcoming resistance to chemotherapy. Recent studies indicate that interactions between leukemia cells and bone marrow (BM) microenvironment confer drug resistance. We here report that SDF-1/CXCR4 and hypoxia in part mediate this resistance. Using the metabolic marker pimonidazole, the hypoxic BM niche in leukemias was found to be greatly expanded, contrary to the discrete, subendosteal or perivascular niches found in normal hematopoiesis. Hypoxia dramatically upregulated expression of the chemokine receptor CXCR4 in AML, thus aiding in homing of LSC in BM niches. CXCR4 is highly prognostic in AML and directly initiates pro-survival signaling through pAKT, pERK, and pSTAT3. Disruption of SDF-1/CXCR4 by the small molecule inhibitors AMD3100 (Plerixafor) or AMD3465 resulted in mobilization of AML cells into circulation, inhibition of intracellular signaling, and greatly enhanced apoptosis induced by chemotherapy or STIs. This concept is presently being tested in clinical trials. BM hypoxia also promotes a switch to glycolytic metabolism and thus contributes to chemo-resistance in the BM niches. These events are in part mediated via transcription factor HIF-1α. HIF-1α and its target gene CAIX were detected in primary acute leukemia BMs, while it was sparingly expressed in normal hematopoietic BM cells. HIF-1α could be induced under hypoxic conditions in co-cultures with BM-derived stromal cells (MSC) through mTOR and MAPK. Silencing of HIF-1α in ALL with siRNA or blockade of mTOR signaling with rapamycin derivatives reduced expression of the glucose transporter Glut-1, diminished glucose flux, decreased glycolytic rate and ATP production, and sensitized leukemic cells to pro-apopotic effects of chemotherapy under hypoxic conditions. A hypoxia-activated pro-drug (PR-104) resulted in cures of 4/5 NOD/Scid/IL2Rγ-KO mice transplanted with primary human leukemia. Altogether, these findings strongly support a role of the hypoxic BM microenvironment in chemoresistance of leukemias and provide a mechanism-based rationale for the elimination of therapy-resistant BM-resident leukemia cells.

355 Inhibition of Vasculogenesis, But Not Angiogenesis, Prevents the Recurrence of Head and Neck Tumors and Glioblastomas Following Irradiation

J. Martin Brown, Mitomu Kioi, G-One Ahn

Department of Radiation Oncology, Stanford University, Stanford, CA

Tumor blood vessels can derive from two sources: by angiogenesis (the sprouting of endothelial cells from nearby blood vessels) and by vasculogenesis, which is produced by circulating cells. We have proposed that the radiation doses given in radiotherapy will abrogate local angiogenesis and thereby force tumor regrowth to rely on vasculogenesis. This involves the recruitment of proangiogenic circulating cells, many of which are derived from the bone marrow. To investigate this hypothesis, we used the subcutaneously- implanted FaDu human head and neck tumor and an orthotopic brain tumor using human U251 GBM cells.

We found that local tumor irradiation induced the influx of bone marrow-derived cells (BMDCs) in a dose-dependent manner into both tumor models, with most of this increase reflecting influx of CD11b+ myelomonocytes. We postulated that this influx of BMDCs was stimulated by increased tumor hypoxia and HIF-1 levels caused by radiation damage to the tumor vasculature. To determine HIF-1 activity in real-time in our brain implanted U251 GBM, we stably expressed the HIF-1 reporter construct 5HRE-luc in U251 cells and showed that HIF-1 activity increased rapidly starting at about 2 weeks following 15 Gy, in parallel to the increase in tumor hypoxia that we observed at this time.

To test our hypothesis that the increased HIF-1 levels were responsible for the increased influx of CD11b+ cells into the tumors, we used the HIF-1 inhibitor NSC-134754. When this was given daily for 2 weeks starting immediately following irradiation, the increased tumor levels of CD11b+ monocytes observed after 15 Gy was abrogated. This treatment also prevented the regrowth of the irradiated tumors following irradiation.

As a further test of our hypothesis, we determined the effect of inhibiting the interaction of SDF-1 with its receptor CXCR4 using the clinically-approved drug AMD3100, which we infused starting immediately following irradiation. This had no effect on the growth of unirradiated tumors in the brain but completely inhibited the recurrence of the irradiated tumors following either a single dose of 15 Gy or the more clinically relevant scheme of 5 daily doses of 2 Gy. Inhibition of angiogenesis with DC101 increased tumor growth delay but did not prevent tumor recurrences.

We also showed that the recurrence of FaDu tumors locally irradiated with 20 Gy was prevented using a monoclonal neutralizing antibody against CD11b+ monocytes. This treatment does not increase the radiosensitivity of normal skin, thereby providing a therapeutic gain.

356 Breast Cancer-Targeted Therapy by Endostatin-Cytosine Deaminse Fusion Protein

Chun-Te Chen[1], Gabriel N. Hortobagyi[2], **Mien-Chie Hung**[1]

Departments of [1]Molecular and Cellular Oncology and [2]Breast Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX

A myriad of anti-angiogenic agents has been developed since the late Judah Folkman discovered the role of angiogenesis in tumor development in the early 1970s. For example, bevacizumab (Avastin) is the first drug of its kind that was approved by the FDA as an anti-angiogenic factor for treating metastatic colorectal, nonsmall cell lung, breast, and glioblastoma cancer patients. However, recent reports indicate that anti-angiogenesis therapy leads to the progression of tumors by increasing invasion and metastasis. Thus, these anti-angiogenesis drugs, though effective in benefiting cancer patients, are not yet perfect. The limited efficacy of bevacizumab might partly be due to its cytostatic nature (i.e., it does not actually kill cells [cytotoxic] but rather limits cell proliferation and growth). Endostatin, a naturally-occurring anti-angiogenic factor, is able to constrain tumor growth by inhibiting growth of new blood vessels. However, endostatin itself only possesses a cytostatic activity similar to bevacizumab.

Previously, we developed a fusion gene construct, Endostatin-Cytosine Deaminse (Endo-CD), containing “cytostatic” endostatin, which retains its tumor targeting ability and a “cytotoxic” protein, cytosine deaminase (CD), that can convert the pro-drug 5-fluorocytosine (5-FC) into the chemotherapeutic drug 5-fluorouracil (5-FU). Endostatin recognizes avb3 integrin, a cell surface receptor of tumor endothelial cells and has an anti-angiogenic activity by inhibiting endothelial cell growth and accordingly resulting in suppression of tumor blood vessels. Thus, Endo-CD is a dual-targeting fusion protein: not only does it have capabilities of limiting cell growth (cytostaticity) and killing cells (cytotoxicity), it is also able to specifically target tumor sites. CD is brought to tumor sites by its fusion with Endostatin, and therefore, 5-FC is converted to cytotoxic 5-FU only at the tumor site. We have encouraging preclinical results that suggest that the therapeutic efficacy of Endo-CD protein is comparable or even better than bevacizumab. In addition, Endo-CD does not produce the toxicity associated with bevacizumab. Furthermore, the half-life of Endo-CD fusion protein is much longer than that of endostatin alone, which is a major setback for moving endostatin into clinical trials. Thus, the Endo-CD fusion protein therapy may be worth moving forward into clinical trials compared with the FDA-approved bevacizumab.

357 In Vitro and In Vivo Efficacy of the Dual PI3-Kinase/mTOR Inhibitor NVP-BEZ235 in Renal Cell Carcinoma

Daniel C. Cho[1], Matthew B. Cohen[1], David J. Panka[1], Rupal Bhatt[2], Michael Collins[2], Sabina Signoretti[2], Michael B. Atkins[1], James W. Mier[1]

[1]Division of Hematology and Oncology, Beth Israel Deaconess Medical Center; [2]Department of Pathology, Brigham and Women's Hospital

Introduction: Inhibitors of mTORC1 have shown activity in metastatic renal cell carcinoma (RCC). As the PI3-K pathway activates numerous kinases and other proteins associated with cell growth in addition to mTOR, disruption of this pathway upstream of mTOR may be more effective than inhibition of mTORC1 alone. To investigate this possibility, the in vitro and in vivo efficacy of the dual PI3-K/mTOR inhibitor NVP-BEZ235 was compared with rapamycin in RCC cell lines.

Experimental Design: Five RCC cell lines (786-0, 769-P, A498, Caki-1, Caki-2) were studied. A NVP-BEZ235 dose of 250nM was chosen for studies based on the complete inhibition of Akt (Thr308) phosphorylation. RCC cells were then exposed to DMSO or treated with rapamycin (100nM) or NVP-BEZ235 and either lysed after 24 hours for western blot analysis or assayed for proliferation by MTS assay after 48 hours. Growth media was isolated and analyzed for VEGF levels by ELISA. HIF activity in treated cells was determined by transfection of an HIF-responsive luciferase construct. Xenografts were generated from 786-O cells in nude beige mice and grown to 10mm in size. Mice were then treated with either saline, rapamycin (2.5mg/kg), or NVP-BEZ235 (40mg/kg) once daily by gavage for 21 days.

Results: Treatment of RCC cell lines with NVP-BEZ235 resulted in greater reduction in cell proliferation and more complete suppression of Cyclin D1 and HIF2α levels than either rapamycin or DMSO. These effects correlated with reduced phosphorylation of eIF4E and Mnk1. Despite suppression of cap-dependent translation suggested by the decrease in p-eIF4E, NVP-BEZ235 did not consistently suppress VEGF expression at either the mRNA or protein level more effectively than rapamycin. Reduction of HIF2α levels correlated with reduced HIF activity by luciferase assay. NVP-BEZ235 induced tumor regression in 786-O xenografts that was associated with inhibition of Akt and S6 phosphorylation. In contrast, rapamycin induced only minimal growth arrest.

Conclusion: Dual inhibition of PI3-K/mTOR with NVP-BEZ235 induced growth arrest in RCC cell lines both in vitro and in vivo more effectively than inhibition of mTORC1 alone. This growth inhibition was associated with reduction of markers of cap-dependent translation and HIF activity in vitro. Variable effects of NVP-BEZ235 on circulating VEGF and intracellular VEGF mRNA levels require studies into mRNA stabilization. Dual inhibition of PI3-K and mTOR may be a promising strategy for the treatment of RCC.

358 A Novel Preclinical Model of Non-Small Cell Lung Cancer Progression and Metastasis for Direct Clinical Translation

Chi-Ping Day[1], John Carter[2], Carrie Bonomi[2], Melinda Hollingshead[3], Glenn Merlino[1]

[1]Laboratory of Cancer Biology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD; [2]Developmental Therapeutics Program, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD; [3]Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI-Frederick, Frederick, MD

The low predictive power of currently available preclinical models, including the prevailing subcutaneous human xenograft models, can be explained in part by their failure to adequately recapitulate the clinical setting. For example, immunocompromised mice can only reconstitute highly aberrant tumor-host interactions, and tumor growth delay has extremely limited value as a relevant endpoint. To begin to overcome these problems, we have developed a preclinical model in which we attempt to more accurately recapitulate the experience of patients with progressive non-small cell lung cancer (NSCLC). Lewis Lung Carcinoma (LLC) tissue, which had been propagated only through serial transplantation in C57BL/6 mice, was stably labeled using a lentivirus encoding a luciferase-GFP fusion gene. LLC tissue was inoculated subcutaneously in syngeneic mice and then resected upon reaching a predetermined size. Following resection, mice were treated with paclitaxel in a setting similar to post-surgery adjuvant chemotherapy in human patients. Bioluminescence imaging was used to monitor the onset and progression of pulmonary metastasis. Paclitaxel at specific dose, which was previously found to have limited effects on subcutaneous tumor growth, effectively suppressed metastasis in lungs by delaying onset and reducing the number of lung nodules. Our results, confirmed using various classes of drug, demonstrated that the response of lung metastases to chemotherapy was distinctive from that of subcutaneous tumors. This model system, which could be completed in a 3- to 4-week period, also revealed roles for the tissue environment and for the importance of metastasis initiation in the efficacy of anti-cancer drugs.

359 Sensitization of Pancreatic Cancer Cells via Inhibition of APE1/Ref-1, a DNA Base Excision Repair and Redox Signaling Protein: Novel Target and Small Molecule Development

Melissa L. Fishel[1], **Mark R. Kelley**[2]

[1]Department of Pediatrics, Section of Hematology/Oncology, Herman B. Wells Center for Pediatric Research, [2]Department of Pharmacology and Toxicology, Department of Biochemistry & Molecular Biology, Indiana University School of Medicine, Indianapolis, IN

The DNA base excision repair (BER) pathway is responsible for the repair of DNA damage caused by oxidation/alkylation agents and ionizing radiation (IR). Removal of the damaged base creates a baseless site that is acted upon by APE1/Ref-1. In addition to APE1/Ref-1's repair role, it also functions as a reduction-oxidation (redox) signaling factor to modify and activate transcription factors such as HIF-1a, NFkB, CREB, AP-1, p53, and others. These transcription factors control the expression of genes important for cell survival, cancer promotion, progression, and angiogenesis linking APE1/Ref-1 protein to these pathways. APE1/Ref-1 has been shown to have an altered level of expression in a variety of cancers including pancreatic, breast, prostate, gliomas, and others. High APE1/Ref-1 expression has also been associated with chemoradiotherapy poor outcome, poor complete response rate, shorter local relapse-free interval, poorer survival, and high angiogenesis.

Pancreatic cancer is a deadly disease that is virtually never cured, and reduction-oxidation (redox) signaling systems have been identified as important targets for pancreatic cancer. Additionally, pancreatic cancers are hypoxic tumors that respond poorly to existing chemotherapeutic agents and radiation. Anti-angiogenic agents, including bevacizumab are under investigation in the treatment of pancreatic cancer. We will present data demonstrating the importance of APE1/Ref-1's redox function in pancreatic cancer cells and as an anti-angiogenesis target using a small molecule inhibitor of APE1/Ref-1's redox function. This data includes evidence that our redox inhibitor of Ape1/Ref-1 (a) blocks APE1/Ref-1 redox, but not its repair function, (b) has single agent growth inhibitory effects on pancreatic cancer cell lines and in xenografts, (c) acts synergistically when utilized in combination with a APE1/Ref-1 DNA repair inhibitor (CI values < 0.5), (d) alters cell cycle kinetics of pancreatic cancer cells, and (e) has anti-angiogenic effects in a variety of angiogenesis assays and is competitive and synergistic with bevacizumab. Additional PK and toxicology data will be presented. These results demonstrate that blocking the redox function of APE1/Ref-1 may have a single agent efficacy in pancreatic cancer with additional uses in other cancers due to its developing anti-angiogenic effects.

360 A Pilot Trial of Pre-Operative (Neoadjuvant) Letrozole in Combination With Bevacizumab in Post-Menopausal Women With Newly Diagnosed Estrogen and/or Progesterone Receptor Positive Breast Cancer

Andres Forero-Torres[1], Mansoor N. Saleh[2], Janice A. Galleshaw[2], Cheryl F. Jones[2], Jatin J. Shah[1], Ivor J. Percent[1], Lisle M. Nabell[1], John T. Carpenter[1], Carla I. Falkson[1], Helen Krontiras[1], Marshall M. Urist[1], Kirby I. Bland[1], Jennifer F. De Los Santos[1], Ruby F. Meredith[1], Valerie Caterinicchia[1], Wanda K. Bernreuter[3], Janis P. O'Malley[3], Yufeng Li[1], Albert F. LoBuglio[1]

[1]Comprehensive Cancer Center, University of Alabama at Birmingham; [2]Georgia Cancer Specialists, Atlanta, GA; [3]Department of Radiology, University of Alabama at Birmingham

Purpose: Tumor content or expression of vascular endothelial growth factor (VEGF) is associated with impaired efficacy of anti-estrogen adjuvant therapy. We designed a pilot study of neoadjuvant letrozole and bevacizumab (anti-VEGF) to assess feasibility and short-term efficacy in post-menopausal women with stage II/III, ER/PR positive breast cancer.

Patients and Methods: Patients were treated with a neo-adjuvant regimen of letrozole, 2.5 mg/day (PO) and bevacizumab 15 mg/kg every 3 weeks (IV) for a total of 24 weeks prior to surgical treatment of their breast cancer. Patients were followed for toxicity at 3-week intervals and tumor assessment (physical exam and tumor ultrasound) at 6-week intervals. PET scans were carried out prior to therapy and 6 weeks after initiation of therapy.

Results: Twenty-five evaluable patients were treated. The regimen was well tolerated except for two patients who were taken off-study for difficult to control hypertension. Objective clinical response occurred in 17/25 patients (68%), including 16% CR and 52% PR. The four patients with clinical CR had pathologic CR in their breasts (16%), although one had residual tumor cells in axillary nodes. Some 8/25 patients (32%) attained stage 0 or 1 status. PET scan response at 6 weeks correlated with clinical CR and breast pathologic CR at 24 weeks ($p < 0.0036$).

Conclusion: Combination neoadjuvant therapy with letrozole and bevacizumab was well tolerated and resulted in impressive clinical and pathologic responses. The Breast Cancer Translational Research Consortium has an ongoing randomized phase II trial of this regimen in this patient population.

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361 Agent Intervention – Protein Therapeutic – Soluble Dll4

Parkash Gill

University of Southern California, Norris Cancer Center, Los Angeles, CA

During angiogenesis, branching of arterial and venous components is orchestrated such that the capillaries from these two compartments fuse in symmetry, anchored in place by interaction with matrix proteins. Vascular endothelial growth factor (VEGF) is indispensable for the formation of primary vascular network and secondary angiogenesis. VEGF, however, requires the presence of precise quantities of several other constituents within well-defined temporal and spatial constraints to construct and remodel the vascular system. Specifically, the Notch signaling pathway is necessary to provide signals for phenotypic determination of arteries and veins and regulated vessel migration and branching leading to the vascular morphogenesis and remodeling In mammals, the Notch family of proteins is composed of four single-pass transmembrane receptors (notch1–4) and five membrane-bound ligands (jagged1, 2, and Dll1, 3, and 4). The Notch receptor-ligand functions through cell-cell interaction. Notch receptor activation requires cleavage of Notch intracellular domain (NICD) and translocation to the nucleus and activation of target genes. Differentiation of vascular cells to arterial or venous is regulated by the differential and restricted expression of a number of genes in arterial or venous endothelial cells. Among these genes are NOTCH1, NOTCH4, and DLL4. Vascular expression of Dll4 and its cognate receptors Notch1 and Notch4 is restricted to arterial endothelium is one of the earliest genes expressed in arterial endothelial cells, is induced by VEGF-VEGFR signaling, and is essential for establishment of the arterial endothelial cell fate. Haploinsufficiency of Dll4 is embryonic lethality due to defects in vascular development, with augmented venous phenotypes, reduced arterial lumen, and premature fusion among the arterial and venous compartment. We have investigated the role of Dll4 in vascular remodeling at sites of angiogenesis, including tumor vasculature. We show that loss of Dll4 function promotes endothelial cell migration, excessive vascular network formation, and reduction in pericyte recruitment, both in embryos and adult mice using Dll4 knockout mouse lines and/or soluble decoy forms of Dll4. Soluble Dll4 has also been tested in vivo in a number of human tumor xenograft models and spontaneous tumor models with remarkable activity (tumor growth inhibition varying from 60 to 80%) and synergy in combination with other targeted therapeutics. Tumor vessels show increased number of vessels that often lack lumen and perfusion and fail to recruit pericytes. Soluble Dll4 is thus a candidate for clinical investigation.

362 Validating GRM1 as a Therapeutic Target in Patients With Melanoma

James S. Goydos[1], Joseph Chan[1], Suzie Chen[2]

[1]Department of Surgery, UMDNJ-Robert Wood Johnson Medical School; [2]Rutgers, The State University of New Jersey

In melanoma, activation and suppression of specific pathways has been implicated in tumor formation, local progression, and metastatic spread. Therapies that target specific components of these pathways have been developed, and many are currently in human trials. However, early results from the use of single agents targeting specific pathway components have been disappointing. It is now generally believed that multiple pathways, many with redundant downstream effects, are dysregulated in melanoma. Furthermore, inhibiting individual pathways often results in unintended feedback reactions that limit the usefulness of these therapies. Targeting multiple pathways or multiple components of key signaling pathways will likely be needed. Recently, our group discovered that ectopic expression of the metabotropic glutamate receptor 1 (GRM1) is oncogenic when ectopically expressed in melanocytes in vitro and in vivo. We demonstrated that targeted murine GRM1 expression in melanocytes in mice is sufficient to induce spontaneous melanoma development with 100% penetrance. Furthermore, we found that the majority of human melanoma tumors and cell lines express GRM1, suggesting that GRM1 may be involved in the oncogenesis of many human melanomas. Indeed, inhibition of GRM1 expression or function suppresses melanoma cell growth, promotes apoptosis, and decreases tumorigenesis in vivo. Importantly, pharmacologic depletion of the GRM1 ligand, glutamate, with the glutamate antagonist Riluzole suppresses melanoma cell growth, increases apoptosis in vitro, promotes apoptosis, and reduces melanoma tumorigenesis in vivo in mice and in patients with melanoma in a preliminary Phase 0 trial. We, therefore, hypothesize that disruption of GRM1 signaling is a novel therapeutic target for the treatment of patients with melanoma, and we have begun to translate this finding into the clinic by performing a Phase II trial of single-agent Riluzole in patients with advanced melanoma. This trial is open and actively accruing patients. We have continued our preclinical studies and have now shown that Riluzole acts mainly through suppression of signaling through the PI3K/AKT pathway, and we have begun examining therapies that combined Riluzole with other agents that suppress signaling through key pathways in melanoma. We now report that the combination of Riluzole and the Raf-inhibitor Sorafenib and the combination of Riluzole and the mTORC-inhibitor Rapamycin are synergistic in inhibiting melanoma proliferation in vitro and in vivo. These findings will now be translated into the clinic in the form of combination trials in patients with advanced melanoma.

363 Methionine Aminopeptidase-2 Interferes With VHL to Regulate VEGF Transcription Through HIF-1 Alpha: Role for Angiogenesis Inhibition

Su Guan[1,2], **Oliver Bogler**[1,2,3], WK Alfred Yung[1,2], Dimpy Koul[1,2]

[1]Neuro-Oncology, [2]Brain Tumor Center, [3]Neurosurgery, The University of Texas M. D. Anderson Cancer Center, Houston

Methionine aminopeptidases (MetAPs) are metalloenzymes involved in the cotranslational removal of protein initiator methionine. At the protein level, co-translational removal of Met and processing of the N-termini is an essential event for cellular viability and the normal functioning of proteins. MetAP2 plays an important role in the proliferation of endothelial cells and cell transformation and has been found in multiple cancers at both the mRNA and protein level. Elevated expression of MetAP2 protein is associated with a poor prognosis in colorectal cancer patients. Suppression of human MetAP2 using anti-sense oligonucleotide resulted in the inhibition of endothelial proliferation and induced apoptosis of tumor cells. In addition, a recent study suggested that MetAP2 inhibitors effectively decreased liver tumor growth. Methionine aminopeptidase-2 (MetAP2) has been pharmacologically linked to cell growth, angiogenesis, and tumor progression, making this an attractive target for cancer therapy. However, it still remains unclear whether MetAP2 plays a similarly important role in GBM. CGAP SAGE Genie database showed that MetAP2 correlated very well with glioma progression, and high expression of MetAP2 was observed in human glioma cells. To study the role of MetAP-2 in glioma angiogenesis, we generated MetAP-2 knockdown SNB19 and LN751 glioma cells that express high levels of MetAP2 using lentiviral Sh RNA against MetAP-2. MetAP2 knockdown cells were less proliferative and induced significant G1 cell cycle arrest in comparison to the parental cells. MetAP-2 knockdown cells were less tumorigenic as shown by colony formation assay. In vivo experiments with shMetAP-2 cells showed increased median survival in comparison to parental cells. We also examined the effect of MetAP-2 on angiogenesis and observed that knocking down of MetAP-2 decreased the VEGF secretion and expression at mRNA and protein level. Decrease in VEGF correlated very well with tube formation assay, as demonstrated by decreased vessel formation by MetAP-2 knockdown cells. To further study the mechanism underlying MetAP2 downregulating VEGF, we used dual-luciferase reporter driving different length of VEGF promoters and performed a luciferase assay. A decrease in luciferase activity was observed ,and the repression of VEGF promoter activity was mainly through HIF-1 alpha. HIF-1 alpha is degradated mainly by von Hippel-Lindau (VHL) and MetAP-2 regulating VHL activity and thereby affecting HIF-1 alpha and VEGF, which will be discussed. In this current study, we have identified the MetAP2-specific substrates that may serve as candidates for clinical assay development.

364 A Phase 1 Trial of Repeat Intrapleural Adenoviral Interferon-Beta in Malignant Mesothelioma and Malignant Pleural Effusion Patients

Andrew R. Haas, Daniel H. Sterman, Edmund Moon, Adri Recio, Steven M. Albelda

University of Pennsylvania Medical Center, Philadelphia, PA

In a previous study, we demonstrated that a single intrapleural dose of an adenoviral vector expressing interferon-ß (Ad.IFN-ß)ß in malignant pleural mesothelioma (MPM) or malignant pleural effusion (MPE) patients was well tolerated and resulted in gene transfer, significant humoral immune responses, and some clinical responses. Based on preclinical data demonstrating enhanced efficacy with multiple Ad.IFN-ßß doses, this Phase I trial was conducted to determine the safety, gene transfer efficiency, and immunologic and clinical responses of two intrapleural Ad.IFN-ß vector (BG00001) doses. Seventeen patients (10 with MPM, 7 with MPE) received two Ad.IFN-ß doses administered through an indwelling pleural catheter in doses ranging from 3 x 1011 to 3 x 1012 viral particles (vp). Subjects were evaluated for toxicity, generation of adenoviral neutralizing antibodies (Nab), gene transfer, humoral immune responses, and tumor responses via 18-fluorodeoxyglucose (18FDG) positron-emission tomography (PET) scans and chest CT scans. Repeat intrapleural Ad.IFN-ß doses were generally well tolerated. One patient with a pre-existing pericardial effusion developed pericardial tamponade. No MTD was reached. Intrapleural IFN-ß expression was detected in most patients after the first dose; however, IFN-ß levels were markedly lower after the second vector dose delivered either 2 weeks (13 patients) or 1 week (4 patients) after the first dose. This lack of expression correlated with rapid Nab induction in nearly every patient. Strong humoral responses against known and unknown tumor antigens were induced in all patients. Several of the patients (4/13) had meaningful clinical responses (mixed and or partial responses) as measured on pre-and post Ad.IFN-ßßi nfusion PET/CT imaging. In conclusion, repeat intrapleural Ad.IFN-ßß doses were safe and induced immune and clinical responses in MPM and MPE patients. Rapid Nab development prevented effective gene transfer after the second dose even with a 7-day dose interval. A current trial delivers a second intrapleural Ad.IFN-ßß vector 3 days after the first dose and in combination with chemotherapy.

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365 Phase I Trial of a Combination of the Multikinase Inhibitor Sorafenib and the Farnesyltransferase Inhibitor Tipifarnib in Advanced Malignancies

David S. Hong[1], Said M. Sebti[2], Robert A. Newman[3], Michelle A. Blaskovich[2], Lei Ye[4], Robert F. Gagel[4], Stacy Moulder[5], Jennifer Wheler[1], Aung Naing[1], Nizar Tannir[6], Chaan Ng[7], Steven Sherman[4], Adel K. El Naggar[8], Rabia Khan[1], Jonathan Trent[9], John Wright[10], Razelle Kurzrock[1]

[1]Department of Investigational Cancer Therapeutics, M. D. Anderson Cancer Center, Houston, TX; [2]Drug Discovery Program, H. Lee Moffitt Cancer Center & Research Institute and Department of Oncologic Sciences, University of South Florida, Tampa, FL; Departments of [3]Experimental Cancer Therapeutics, [4]Endocrine Neoplasia & Hormonal Disorders, [5]Breast Medical Oncology, [6]GU Medical Oncology, [7]Radiology, [8]Pathology, and [9]Sarcoma, M. D. Anderson Cancer Center, Houston, TX; [10]Cancer Therapy Evaluation Program, National Cancer Institute, Bethesda, MD

Purpose: We evaluated the safety, maximum tolerated dose, pharmacokinetics, and biologic effects of the combination of the Raf-1, RET, KIT, platelet-derived growth factor receptor (PDGFR) and VEGFR2 kinase inhibitor sorafenib and the farnesyltransferase inhibitor tipifarnib.

Experimental Design: A standard 3+3 phase I dose-escalation design was used with a 28-day cycle (sorafenib daily and tipifarnib for 21 days, by mouth).

Results: Fifty patients were treated; 43 reached restaging evaluation after cycle two. The most common side effects were grade 1–2 rash, hyperglycemia, and diarrhea. Dose-limiting toxicity was rash, and the recommended phase II dose is sorafenib 400 mg po qam/200 mg po qpm and tipifarnib po 100 mg BID. Despite the low doses of tipifarnib, one-quarter of patients had ≥50% reduction in farnesyltransferase (FTase) levels. Interestingly, six of eight patients with medullary thyroid cancer (MTC) had durable stable disease (N=3) or partial remissions (N=3) lasting 12 to 26+ months. Five of the six responders had available tissue, and RET gene mutations were identified in them. Prolonged ≥6 months) stable disease was also seen in nine patients as follows: papillary thyroid cancer (N=4; 18+ to 27+ months); adrenocortical cancer (N=2; 7 and 11 months); and one each of melanoma (PDGFR mutation-positive) (14 months), renal (6 months), and pancreatic cancer (6 months).

Conclusion: Our study shows that the combination of tipifarnib and sorafenib is well tolerated. Activity was seen, especially in patients with medullary thyroid cancer, a tumor characterized by RET mutations.

366 A Phase I Trial of ABT 510 Administered Concurrently With Temozolomide Chemo-Radiotherapy in Patients With Newly Diagnosed Glioblastoma Multiforme

Manasi S. Kekan, John B. Fiveash, James M. Markert, **G. Yancey Gillespie**, Hui-Chien Kuo, Sreelatha Meleth, Candece L. Gladson, L. Burt Nabors

UAB Brain Tumor SPORE, University of Alabama at Birmingham

We have completed a Phase I dose-escalation trial to define the maximum tolerated dose (MTD) of ABT-510 when used concurrently with temozolomide (TMZ) and radiotherapy (RT) in patients with newly diagnosed glioblastoma multiforme (GBM). ABT-510 (Abbott Laboratories, Abbott Park, IL, USA) is a Thrombospondin-1 (TSP-1) mimetic drug with anti-angiogenic properties. Our preclinical data demonstrated the synergistic anti-tumor properties of ABT510 with irradiation in both in vitro and in vivo mouse models of gliomas. Twenty-three patients with newly diagnosed, histologically-verified GBM were enrolled between April 2005 and January 2007, after obtaining written consent. The study was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board. Four cohorts with three patients in each receiving subcutaneous ABT 510 injection at doses of 20, 50, 100, and 200 mg/day were studied. The starting dose was primarily based on preclinical findings from animal studies and Phase I studies on healthy subjects and cancer patients. Treatment plan included a 10-week induction phase (TMZ and RT with ABT 510) followed by a maintenance phase (ABT-510 and TMZ) of 14 cycles, each consisting of 28 days. Patients were monitored with brain MRI along with laboratory values for dose limiting toxicities (DLT) defined as grades 3–4 non-hematological toxicities and grade 4 hematological toxicities (neutropenia or thrombocytopenia). In the absence of a DLT in at least two of the three patients, the dose was escalated by 50% in the next cohort of patients. Therapy was discontinued if 14 maintenance cycles were completed, disease progression occurred, or if the patient requested withdrawal. Disease progression and survival statistics are presented here. During this trial, grade 3/4 DLTs were not observed, even after the dose was increased to 200 mg/day. Thus, the last cohort was expanded to include 14 patients. An MTD was not defined. The median time to tumor progression (TTP) was 220 days, and the median overall survival was 422 days. Gene expression analysis of the tumor pathology was performed to evaluate the relationship between the expression of TSP-1, TSP-2, and patient response to the drug. In summary, ABT 510, at subcutaneous doses up to 200 mg/day, is tolerated well with concurrent TMZ and RT in patients with newly diagnosed GBM.

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367 Progesterone and Bevacizumab in Targeted Molecular Therapy for Endometrial Cancer

Victoria P. Korovkina[1], Suzy Davies[2], Kimberly K. Leslie[1]

[1]Department of Obstetrics and Gynecology, The University of Iowa, Iowa City; [2]Department of Obstetrics and Gynecology, University of New Mexico Health Sciences Center, Albuquerque

Endometrial cancer is the most frequent gynecologic cancer in women. Long-term outcomes for patients with advanced stage or recurrent disease are poor. Progesterone inhibits growth of endometrial cancer through its receptors A (PRA) and B (PRB). The receptors are present in nuclei of endometrial carcinomas; however, expression of only one isoform is common. The abnormal PRA/PRB ratio may lead to loss of differentiating effects of progestins and render hormonal therapy ineffective. Interventions that target progesterone-controlled pathways of endometrial differentiation downstream from PR have the potential to overcome limitations of hormonal therapy that requires robust expression of PR. Our data indicate that progesterone acutely (in the first 12 h) downregulates the vascular endothelial growth factor (VEGF), the platelet derived growth factor (PDGF), and the signal transducer and activator of transcription 3 (STAT3), resulting in inhibition of angiogenesis and proliferation. Thus, targeted therapy against any of these molecules may mimic the effects of progesterone independent of PR. Bevacizumab (Avastin, a humanized monoclonal antibody against VEGFA) is a new therapeutic option for patients with endometrial cancers that is now under study by the Gynecologic Oncology Group in a phase 2 trial, GOG 229E. Using a xenograft model of endometrial cancer we show that bevacizumab prevents tumor growth in some animals and limits tumor size in animals that do develop cancer. We have identified genes and pathways that confer sensitivity to bevacizumab, among them PKB/Akt, S6K, MSK1, and PKC delta. Alternatively, pathways that may contribute to resistance to this agent include c-JUN, MEK ½, GSK-3 alpha, raf-1, and SMAD. Interestingly, unlike progesterone that downregulates STAT3, bevacizumab activates STAT-3 pathway, and this mechanism may in part explain resistance to bevacizumab-based therapy. Genes and pathways that are differentially regulated by progesterone and/or bevacizumab may be important targets for future therapeutic interventions.

368 Combining Anti-Invasive and Anti-Angiogenic Therapies for the Treatment of GBM

Laura Lewis-Tuffin[1], Bernd Scheithauer[2], Caterina Giannini[2], Kurt A. Jaeckle[1], Jan C. Buckner[2], Evanthia Galanis[2], Jann N. Sarkaria[2], **Panos Z. Anastasiadis**[1]

[1]Mayo Clinic Florida, Jacksonville, FL; [2]Mayo Clinic Rochester, Rochester, MN

Anti-VEGF antibody therapy with bevacizumab provides significant clinical benefit and is increasingly becoming the standard of care for patients with recurrent glioblastoma multiforme (GBM). Unfortunately, progression on bevacizumab therapy in a subset of patients is associated with an aggressive, diffuse, multi-focal disease recurrence pattern and a short subsequent survival interval. Using a novel primary human GBM xenograft model, we have reproduced a similar phenotype in which bevacizumab therapy results in increased glioma invasiveness and in a multi-focal disease recurrence pattern. Our preliminary data also suggest that glioma invasion is critically controlled by Src- and PI3-kinase-dependent signaling pathways. We find that Src activation is highest at the leading infiltrating edges of intracranially injected GBM tumors, with corresponding increases in the tyrosine phosphorylation of the Src substrates p120 catenin, p130cas, and Vav2 at these regions. Consistent with the role of these Src substrates on the regulation of Rho family GTPases, which are critically involved in the reorganization of the actin cytoskeleton during cell migration, we find that the downstream Rho signaling target cofilin is also phosphorylated at the invasive tumor front. We postulate that the increased Src activation at the invasive front is due to local tumor hypoxia that is further exacerbated by anti-angiogenic therapy. Furthermore, data from both conventional GBM cell lines and human GBM xenografts in short-term culture suggest that PI3K signaling cooperates with Src family kinases in promoting GBM cell migration and invasiveness. Based on these data, we hypothesize that the increased invasiveness associated with anti-VEGF therapy is due to increased signaling through these pathways. Consistent with this hypothesis, we found that the Src-family kinase (SFK) inhibitor dasatinib can prevent both the increased invasion and the multi-focal disease progression pattern induced by bevacizumab in mouse xenografts. In part based on these preliminary data, we are initiating a clinical trial testing the combination of bevacizumab and dasatinib in patients with recurrent GBM.

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369 Anti-Tumor Activity of an Anti-HDGF Antibody Combined With Gemcitabine and Avastin in Lung Cancer Heterotransplant Models

Li Mao[1], Hening Ren[2]

Department of Oncology and Diagnostic Sciences, The University of Maryland Dental School and University of Maryland Greenebaum Cancer Center, Baltimore

Background: Hepatoma-derived growth factor (HDGF) is a mitogen for endothelial cells, vessel smooth muscle cells, fibroblasts, as well as some epithelial cells. It is overexpressed in a number of human cancers, and its overexpression in tumors strongly correlates with tumor progression, recurrence, and metastasis. We have shown that anti-HDGF antibodies may inhibit growth of nonsmall cell lung cancer (NSCLC) in xenograft tumor models, suggesting that HDGF may be a therapeutic target for lung cancer.

Methods: Four lung heterotransplant tumor models were treated when tumors were fully established (> 500 mg) with 1.2 mg Gemcitabine and 100 µg Avastin with and without 250 µg monoclonal anti-HDGF antibody H3 per animal intra-peritoneal (IP) every 3 days up to 3 weeks. Tumor sizes were measured to compare anti-tumor activities of the treatments.

Results: Gemcitabine combined with Avastin was effective in one of the four models. In the effective model, the treatment resulted in almost complete tumor regression, but tumors quickly returned after stopping treatment. Interestingly, two of the four tumor models were sensitive to the combination plus the anti-HDGF antibody. In both models, the later treatment resulted in nearly complete tumor regression, and the tumors did not return up to 2 months after stopping the treatment.

Conclusion: Our data suggest that the anti-HDGF antibody may be used in combination with currently used regimens to improve treatment effects for patients with NSCLC.

370 Biological Significance of Chemokine Receptor-4 and Focal Adhesion Kinase Silencing in Ovarian Carcinoma

Edna M. Mora[1,2], L.S. Mangala[2], Rebecca L. Stone[2], Alpa Nick[2], Pablo Vivas-Mejias[1,2], Gabriel Lopez-Berestein[2], Anil K. Sood[2]

[1]University of Puerto Rico Comprehenisve Cancer Center and the University of Puerto Rico School of Medicine, San Juan; [2]The University of Texas, M. D. Anderson Cancer Center, Houston

Ovarian cancer has the highest mortality among gynecologic malignancies. Effective treatment of the disease is hampered by its aggressive clinical progression, even after successful initial resection and adjuvant therapy. Therefore, new drugs are needed to effectively treat the disease. RNAi is an emerging technology that is highly efficient in regulating protein targets in vitro and in vivo. Among the many potential targets responsible for ovarian cancer growth and progression, chemokine receptor-4 (CXCR4) and its downstream signaling through the FAK axis is being recognized as an important target. CXCR4 is a G-protein coupled receptor that, upon ligand (CXCL12) binding, activates growth, adhesion, and migration in several cell types. To explore the mechanisms and biological significance of CXCR4/FAK mediated signaling in ovarian carcinoma, we performed in vitro experiments using single and dual inhibition of these proteins using chemically synthesized siRNA. We found that CXCL12 induced activation and internalization of CXCR4. Upon stimulation, CXCR4 rapidly forms a complex with FAK leading to increased phosphorylation of FAK (FAKY397, FAKY861, and FAKY925). Inhibition of CXCR4 or FAK resulted in significantly decreased migration (p= 0.01), invasion (p=0.03), and adhesion (p=0.001) of ovarian cancer cells. Dual inhibition of these targets resulted in at least additive decreases in these functional effects. We are now conducting in vivo experiments to determine the effect of liposomal siRNA on tumor growth, angiogenesis, and metastasis. Our data suggest that concurrent inhibition of CXCR4 and FAK may be a potential strategy for the treatment of ovarian carcinoma.

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371 Phase II Trial of BAY 43-9006 (Sorafenib-BAY) in Metastatic Melanoma

Patrick A. Ott[1], Sara Safarzadeh-Amiri[1], Lauren Goldberg[1], Christina Min[1], Peter Brooks[1], Joanne Yoon, Anne Hamilton[2], Hermann Yee[1], Michael Buckley[1], John Wright[3], Iman Osman[1], Franco Muggia[1], Leonard Liebes[1], Anna C. Pavlick[1]

[1]New York University Cancer Institute, New York University School of Medicine, New York; [2]Sydney Melanoma Unit and University of Sydney, Sydney, Australia; [3]Investigational Drug Branch, National Cancer Institute, Bethesda, MD

Background: The primary purpose of the current study was to explore the efficacy and safety of sorafenib monotherapy in patients with metastatic melanoma. In correlative studies, we investigated the effect of this treatment on the MAPK pathway and downstream components—cyclinD1, p44ERK, and Ki67. We also aimed to assess for an association of BRAFV^{6}00E mutational status and response to sorafenib therapy.

Methods/Eligibility: Patients with biopsy-accessible, untreated, and unresectable melanoma with measurable disease by RECIST criteria. **Treatment:** Sorafenib 400 mg po BID day 1–28 on a 28-day cycle. Patients were re-imaged every two cycles and treated until progression of disease (POD). Follow-up analysis was performed at study completion. Tumor BRAFV600E mutational status was determined by routine PCR sequencing and mutation-specific PCR (MSPCR). Immunohistochemistry (IHC) staining for cyclinD1, p44ERK, and Ki67 was performed on paraffin embedded tissue from day 1 (pre-treatment) and day-28 biopsy samples.

Results: 36 patients (2 IIIC, 11-M1a, 6-M1b, 17-M1c) were enrolled. **Median age:** 64 (range 22–91); LDH > 1.5 x nl in 12. The main toxicities included diarrhea, alopecia, rash, mucositis, nausea, hand-foot syndrome, and intestinal perforation. Three patients had a partial response (PR) lasting 175, 69, and 65 days. Five patients experienced stable disease (SD) with a median duration of 34 weeks. Routine BRAF sequencing yielded 27 wild-type (wt) and 6 mutant tumors, whereas MSPCR yielded 12 wt and 18 mutant tumors. No correlation was seen between BRAF mutational status (as analyzed by either method) and PR/SD. Downregulation of X for cyclinD1, p44ERK, and Ki67 was demonstrable in two patients who had a PR. Paired specimens from day 1 (pre-treatment) and day 28 biopsies were obtained from 15 patients (responders and non-responders), and IHC results for cyclinD1, p44ERK, and Ki67 will be shown at the time of presentation.

Conclusions: Sorafenib monotherapy has no meaningful activity in advanced melanoma patients. BRAFV600E mutational status is detected more frequently by MSPCR compared to conventional PCR sequencing in melanoma. Due to the low number of responses in this trial, no conclusion can be drawn about an association between BRAFV600E mutational status and response to treatment with Sorafenib.

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372 Anti-MMP-9 DNAzyme: A New Therapeutic Agent to Fight Cancer

Tayebeh Pourmotabbed[1,2], Chad Batson[1], Mohammad S. Amiri [1], Miranda Hallett[1], Christopher Duntsch[2], Qihong Zhou[2], Umar Akbar[2]

Departments of [1]Molecular Sciences and [2]Neurosurgery, University of Tennessee Health Science Center, Memphis

Matrix metalloprotinase-9 (MMP-9) is a member of the matrician family of enzymes that are associated with many pro-oncogenic events such as angiogenesis, proliferation and tumor metastasis. The downregulation of MMP-9 expression by siRNA and ribozymes and its inhibition by chemical inhibitors have all led to decreased invasion and colonization of certain cancer cell lines. Batimastat, a synthetic broad spectrum MMP inhibitor, has been shown to reduce growth of primary tumor, onset of distant metastases, and even prolong survival of animals with pancreatic, orthotopic colon, or liver tumors. However, most clinical trials using anti-MMP inhibitors such as Batimastat have failed, most likely due to the significant side effects associated with these drugs. The side effects include but are not limited to myalgia, anorexia, nausea, vomiting, asthenia, and inflammatory polyarthritis. The utilization of anti-MMP all-RNA ribozyme and/or siRNA as a therapeutic agent is limited because of the lack of efficient delivery of these molecules into cells and is also complicated by the need for a carrier, usually a retroviral vector or a lipid-based compound. RNA-cleaving DNA-based enzymes (DNAzymes) are advantageous when compared to small molecule drugs and siRNA because of their specificity, stability, and lack of side effects associated with viral vectors. Here, we show that DNAzymes targeting MMP-9 mRNA (AGBD) inhibit MMP-9 protein expression, cell proliferation, and astrocytoma and glioma cell invasion in vitro. In addition, a single intracranial injection of AGBD is sufficient to reduce the size of intracranial C6 and 9L glioma in rats by 60%; a weekly intratumoral injection reduced the size of mammary tumors generated in a MMTV-PyMT transgenic mouse model by 71%. The tumor reductions correlate with a decrease in the level of MMP-9 in tumor/stroma cells. In situ hybridization in brain and breast tissues demonstrated that they have efficiently taken up the AGBD molecules and that the DNAzyme is stable in these tissues for at least 20 days post-injection. Neurological testing and H&E staining of the normal brain and breast tumor tissue slices suggests that DNAzyme is safe and not associated with significant cytotoxic effects. Given the potential for systemic administration, these results indicate that anti-MMP-9 DNAzyme can be used as a novel therapeutic agent to fight cancer.

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372B Biology of Alpha Particle Immunotherapy

David A. Scheinberg, Michael R. McDevitt, Freddy Escorcia, Jon Seideman, Kurtis Haro, Hui Min Tseng

Memorial Sloan-Kettering Cancer Center

RO1-CA55349: The long-term goals of the program have been to understand the biology of alpha particle immunotherapy. Over the last 17 years on this grant, we have established that biologically active, novel radioconjugates, many based on the anti-CD33 CDR- grafted IgG1 HuM195, can be constructed, characterized, and translated into successful clinical trials in patients with AML. Most recently, we described a novel class of radio-immunopharmaceutical, a targetable atomic nanogenerator with exceptional potency that has entered human clinical phase I use and that already shows anti-leukemia activity at the lowest doses. We are now studying the radiobiology of the tumor and its environment, especially its vasculature, to better understand how to make more effective alpha-emitting agents. Currently we are analyzing (1) several new questions that have emerged out of our previous work targeting vasculature and (2) mechanisms of action and resistance to antibody targeted alpha-particle irradiation. Throughout this work, as before, we aim to ultimately provide therapeutic agents and concepts for their appropriate use and direct application to human clinical trials. The potency and short range of alphas makes them appealing agents for selective killing of tumor neovasculature. Initial studies showed that we could deplete and normalize residual tumor vessels in animal models using alphas targeted to VE-cadherin on neovasculature, with effective anti-cancer activity. We are exploring how to best utilize this finding to optimize combining alpha immunotherapy to neovasculature in combination with agents directed to the tumor cells themselves. We also explore mechanisms by determining how the properties of the subsequent anti-tumor agents affect kinetics and tumor therapy outcomes following vessel normalization. We are also examining potential mechanisms of cellular resistance to alpha particle damage including (a) DNA damage sensors and transducers or apoptosis mediators or cell cycle checkpoints that distinguish alpha-resistant from sensitive cells or affect alpha sensitivity; (b) DNA repair or damage mitigation mechanisms; and (c) radiation resistant leukemia cells. Understanding this critical biology should allow better strategies to avoid normal cell killing and tissue damage, while enhancing tumor cell kill.

373 Preclinical Development of TGF-Beta Antagonists for Cancer Therapy

Lalage Wakefield[1], Yu-an Yang[1], Jeong-Seok Nam[1], Doug Weinberg[1], Dror Luger[1], Masaki Terabe[1], John Morris[1], Jay Berzofsky[1], Scott Lonning[2]

[1]National Cancer Institute, Bethesda, MD; [2]Genzyme Corp, Framingham, MA

Elevated expression of transforming growth factor-beta (TGF-b) correlates with metastasis and poor prognosis for many human tumors. Potential tumor-promoting activities of TGF-b include direct effects on the tumor (enhanced invasion and migration) as well as effects on the stroma (enhanced angiogenesis and immunosuppression). Thus, TGF-b antagonists offer the attractive possibility of simultaneously targeting both the tumor parenchyma and stroma. However, TGF-bs also play important roles in normal homeostasis and in suppressing the early stages of tumorigenesis. Because of these complex roles involving the interplay of many cellular targets, animal models are critical for evaluating interventions that target the TGF-bß pathway. Using the MMTV-Neu transgenic mouse model of metastatic breast cancer, we previously made the surprising observation that an antibody-like TGF-b antagonist could suppress metastasis with remarkably few of the predicted adverse effects, suggesting that it might be possible to selectively antagonize the "bad" TGF-b while sparing the "good." Our current preclinical work is focused on more detailed mechanistic analysis in order to provide information in support of ongoing early phase clinical trials with TGF-b antagonists. Using an anti-TGF-b monoclonal antibody (1D11) in the 4T1 syngeneic transplant model of metastatic mammary cancer, we have shown that therapeutic efficacy involves the combination of many small effects on multiple cellular compartments, mostly occurring locally at the tumor site, a mechanism that we refer to as "Death by a Thousand Cuts." These individually small-magnitude effects synergize to generate a more hostile tumor stroma, primarily through reactivation or unmasking of effective anti-tumor immune responses. The absence of a major effect of 1D11 on any one cell compartment may be critical for a good therapeutic index and the avoidance of autoimmune complications. However, it poses significant challenges for the development of useful biomarkers of efficacy in hitting the molecular target. Work to develop novel combination strategies and to identify critical molecular determinants of desirable versus adverse responses to TGF-b antagonism in vivo is ongoing.

374 TNFalpha: Tumor Friend or Tumor Foe? Translational Potential

Ralph R. Weichselbaum[1], Michael A. Beckett[1], Yuru Meng[1], Hua Liang[1], Harold G. Sutton[1], Kenneth Cohen[2], Helena J. Mauceri[1]

Departments of [1]Radiation and Cellular Oncology, and [2]Medicine, Section of Hematology/Oncology, University of Chicago, Chicago, IL

A gene therapy strategy, based on data from our laboratory, that is currently under clinical investigation employs a replication defective adenovirus encoding the radio-inducible/chemo-inducible DNA sequences of the egr-1 promoter ligated upstream of the cDNA for human TNFα (TNFerade™, GenVec). Phase I and II clinical trials employing TNFerade™ and radiotherapy demonstrated complete pathological responses in esophageal, rectal, and pancreatic cancer and in patients with melanoma and sarcoma. Results of a randomized Phase III trial in locally advanced unresectable pancreatic cancer that compare 5FU+IR to FU+IR+TNFerade™ are encouraging in that the TNFerade™ cohort demonstrates a survival advantage, although the increase in survival has not yet reached significance. We have been investigating TNF-α signaling using mice with germ line deletions in TNFα receptor 1 and 2 implanted with B16-F1 melanoma. We report that treatment with Ad.Egr-TNF+IR inhibits tumor growth compared to IR alone in wild-type but not in receptor-deficient animals (TNFR1,2-/- and TNFR1-/-). Combined treatment with Ad.Egr-TNF+IR produces an increase in tumor-associated endothelial cell apoptosis in wild-type but not in receptor deficient mice, suggesting that the tumor associated endothelium is a target for Ad.Egr-TNF radiosensitization and implicate TNF-α signaling in tumor radiosensitivity. We also report here that depletion of tumor-associated macrophages prior to IR increases IR anti-tumor effects and that co-implantation of tumor cells with BM-derived macrophages (BMDMφ) increases tumor radioresistance. Studies using mice with germ line deletions of TNF receptors 1 and 2 (TNFR1,2-/-) or TNF-α (TNF-/-) show that radioresistance mediated by BMDMφ requires intact TNF-α signaling and that the radioprotective effect of TNFα is mediated by the upregulation of VEGF production in tumor-associated macrophages (TAMφ). Treatment with a soluble TNF receptor fusion protein (Enbrel®) that blocks the effects of TNFα combined with IR enhances tumor regression compared to IR alone. These data provide a mechanistic basis for targeting macrophage populations generally and TNFα-induced macrophage VEGF specifically. These findings suggest that blockade of TNF/TNFR signaling in TAMφ is an attractive target to improve the efficacy of radiotherapy and suggest that macrophage blockade mediates in vivo radiosensitivity predominantly through effects on the tumor microenvironment. These results present two potential therapeutic strategies to modify TNFα and improve radiotherapy.

375 Src Activation Takes Center Stage in Stress-Induced Tumor Growth

Guillermo N. Armaiz-Pena[1], Gabriel J. Villares[1], Yvonne G. Lin[1], Liz Y. Han[2], Angela M. Sanguino[2], Michael T. Deavers[1], Gary G. Gallick[1], Menashe Bar-Eli[1], Gabriel Lopez-Berestein[1], Steve W. Cole[3], Susan K. Lutgendorf[4], **Anil K. Sood**[1]

[1]The University of Texas M. D. Anderson Cancer Center; [2]University of Texas Health Science Center Graduate School of Biomedical Sciences; [3]University of California Los Angeles School of Medicine; [4]University of Iowa

Clinical studies demonstrate that chronic stress, depression, and other behavioral factors influence cancer progression. However, the underlying mechanisms are not fully understood. Src, a non-receptor tyrosine kinase, is a central converging point for many cancer signaling pathways. In this study, we examined the biological and clinical significance of Src in stress mediated tumor growth. Norepinephrine (NE) rapidly activated SrcY419 in ß-adrenergic receptor (ADRB) positive ovarian cancer cell lines (HeyA8 and SKOV3ip1) but not in ADRB-null A2780 cells. Confocal microscopy showed that Src was rapidly recruited to the cellular membrane after NE exposure in ADRB positive ovarian cancer cells. Furthermore, treatment with different ADRB agonists and blockers determined that ADRB2 are required for SrcY419 phosphorylation. Treatment with a cAMP agonist or PKA agonist/antagonists demonstrated that cAMP/PKA signaling is required for NE-induced Src activation. The unexpected tyrosine phosphorylation via cAMP/PKA activation was found to be mediated by direct phosphorylation of SrcS17 following NE treatment. In Src–/– cells transiently expressing Src, NE caused SrcY419 phosphorylation, which was absent in the Src S17A (mutated) cells. Exposure to NE resulted in an increase in ovarian cancer cell migration and invasion that was completely abrogated by Src-targeted siRNA ($P < 0.01$). In an orthotopic mouse model of ovarian carcinoma (HeyA8 and SKOV3ip1), chronic restraint stress significantly increased tumor weights (182 and 315% increase, $P < 0.05$). This increase in tumor growth was completely blocked by Src silencing with Src siRNA-DOPC. To test the clinical significance of our biological findings, we examined 91 epithelial ovarian cancer samples. Elevated pSrcY419 was associated with worse patient survival ($P < 0.001$), high tumoral NE levels ($P < 0.001$), and high scores on the Center for Epidemiologic Studies Depression Scale (CESD; $P = 0.008$). This work is the first to provide a critical link between Src activation and stress-mediated cancer growth.

376 Loss of Imprinting of Insulin Growth Factor II Gene and Risk of Cancer

Marcia Cruz-Correa[1,2,4], Ronghua Zhao[2], Raul Bernabe[2], Mercedes Lacourt[2], Mariana Berho[3], Linda M. Hylind[4], Steven Wexner[3], Hengmi Cui[4], Andrew P. Feinberg[4], Francis M. Giardiello[4]

[1]Departments of Medicine and Biochemistry and [2]Cancer Center, University of Puerto Rico, San Juan, PR; [3]Departments of Pathology and Surgery, Cleveland Clinic Florida, Weston, FL; [4]Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD

Background and Aims: Loss of genomic imprinting (LOI) of insulin-like growth factor II gene (IGF2) involves abnormal activation of the normally silent maternally inherited allele. LOI of IGF2 in peripheral blood lymphocytes (PBL) is associated with personal and family history of colorectal neoplasia, but whether this epigenetic marker is associated with other cancers is unknown.

Methods: A cross-sectional study of consecutive patients undergoing colonoscopy for screening or any medical indication was conducted. RT-PCR for imprinting analysis of IGF2 was performed on normal peripheral blood lymphocytes of these individuals. Patient history of cancer was correlated with LOI expression in PBL. Odds ratios (OR) and 95% confidence ratios were calculated.

Results: The prevalence of LOI of IGF2 was examined in 302 persons. Individuals with LOI of IGF2 in PBL had an OR of 2.04 (95% CI: 1.19–3.53) for a history of any site of cancer compared to those without LOI. In particular, women with LOI had increased risk of breast cancer (OR= 5.98, 95% CI: 1.53–23.4), and those with LOI had increased risk of colon (OR = 3.82, 95% CI: 1.70–8.60) and rectal (OR = 4.38, 95% CI: 1.88-10.2) cancer. LOI was not associated with prostate cancer.

Conclusions: Abnormal imprinting of IGF2 in PBL was associated with any site—breast, colon, and rectal cancer. These findings support the concept that LOI of IGF2 is a germline constitutional epigenetic alteration that marks individuals with increased risk for several human malignancies.

377 Protein Phosphatase 2A Subunit Gene Haplotypes and Proliferative Breast Disease Modify Breast Cancer Risk

William D. Dupont[1], Kathryn S. Poland[2], Joan P. Breyer[2], Kevin M. Bradley[2], Peggy A. Schuyler[1], W. Dale Plummer[1], Melinda E. Sanders[3], David L. Page[3], **Jeffrey R. Smith**[2,4],

Departments of [1]Biostatistics, [2]Medicine, and [3]Pathology, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN; [4]Medical Research Service, VA Tennessee Valley Healthcare System, Nashville, TN

Background: Protein phosphatase 2A (PP2A) is a major cellular phosphatase and plays key regulatory roles in growth, differentiation, and apoptosis. Women diagnosed with benign proliferative breast disease are at increased risk for the subsequent development of breast cancer.

Methods: We evaluated genetic variation of PP2A holoenzyme subunits for potential contribution to breast cancer risk. We performed a nested case-control investigation of a cohort of women with a history of benign breast disease. Subjects were followed for an average of 18 years; DNA prepared from the original archival benign breast biopsy (1954–95) was available for 450 women diagnosed with breast cancer on followup and for 890 of their 900 controls who were matched on race, age, and year of entry biopsy.

Results: Single allele- and haplotype-based tests of association were conducted, with assessment of significance by permutation testing. We identified significant risk and protective haplotypes of PPP2R1A, giving odds ratios of 1.63 (95% CI 1.3–2.1) and 0.55 (95% CI 0.41–0.76), respectively. These odds ratios remained significant upon adjustment for multiple comparisons. Women with both the risk PPP2R1A haplotype and a history of proliferative breast disease had an odds ratio of 2.44 (95% CI 1.7–3.5) for the subsequent development of breast cancer. The effects of haplotypes for two regulatory subunit genes, PPP2R2A and PPP2R5E, on breast cancer risk were nominally significant but did not remain significant upon adjustment for multiple comparisons.

Conclusion: This evidence supports the previously hypothesized role of PP2A as a tumor suppressor gene in breast cancer.

378 Flaxseed Lignan for Chemoprevention in Premenopausal Women at High Risk for Development of Breast Cancer

Carol J. Fabian, Qamar J. Khan, Priyanka Sharma, Susan Baxa, Trina Metheny, Carola M. Zalles, Theresa Phillips, Bruce F. Kimler.

University of Kansas Medical Center, Kansas City

The lignans enterolactone and enterodiol are derived from the action of gut bacteria on ingested Secoisolariciresinol diglycoside (SDG), which is commonly found in flaxseed. Enterolactone and enterodiol are thought to impair mammary carcinogenesis via reduction in aromatase activity and the mid-cycle surge of luteinizing hormone. We assessed the modulatory activity of 1 year of SDG on a number of risk biomarkers for breast cancer in a prospective Phase II pilot study. The primary endpoint was a change in proliferation in benign breast tissue as measured by Ki-67 immunocytochemistry.

Pre-menopausal women age 21–55 at increased risk for breast cancer underwent a baseline random periareolar fine needle aspiration (RPFNA) between the first and 10th days of their menstrual cycle. Those with RPFNA evidence of hyperplasia and Ki-67 $\geq$2% were invited to participate. Women taking flaxseed or oral contraceptives were ineligible. All women took one Brevail® (Lignan Research) capsule containing 50 mg of SDG daily. Ki-67 staining was performed with DAKO M7240 antibody on hematoxylin counterstained slides, and the number of cells staining positive in 500 cells within hyperplastic clusters was counted.

Forty-nine women were enrolled on study between February 2006 and June 2008. Of these, 4 stopped prematurely and 45 (92%) have completed study and undergone followup RPFNA to provide evaluable specimens. Baseline characteristics of the 45 women completing study are as follows: median age 43 (range 29–51), median baseline 5 year Gail model risk 1.6% (range 0.1–5.7%), median Ki-67 4.0% (range 2.0–16.8%). Some 35 % had cytologic evidence of hyperplasia without atypia, and 62% had atypia. At repeat RPFNA, Ki-67 expression was reduced, median value of 2.0% (range 0–15.2%) and median relative reduction of 0.67. Thirty-six of the 45 women (80%; $p<0.001$ by Wilcoxon signed ranks test) demonstrated a decrease in proliferation.

Conclusion: The reduction in proliferation as measured by Ki-67 expression in hyperplastic benign breast tissue after 12 months of 50 mg of SDG administered daily as Brevail® warrants study in a randomized, blinded, placebo-controlled clinical chemoprevention trial.

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379 PALB2 in Pancreatic Cancer Susceptibility: Gene Discovery Through Exomic Sequencing

Siân Jones[1,2], Ralph H. Hruban[2,3], Mihoko Kamiyama[3], Michael Borges[3], Xiaosong Zhang[1,2], D. Williams Parsons[1,2], Jimmy Cheng-Ho Lin[1,2], Emily Palmisano[2], Kieran Brune[2], Elizabeth M. Jaffee[2,3], Christine A. Iacobuzio-Donahue[2,3], Anirban Maitra[2,3], Giovanni Parmigiani[2,3], Scott E Kern[2,3], Victor E. Velculescu[1], Kenneth W. Kinzler[1], Bert Vogelstein[1,2], James R. Eshleman[2,3], Michael Goggins[2,3,4], **Alison P. Klein**[2,3]

[1]Ludwig Center for Cancer Genetics and Therapeutics and Howard Hughes Medical Institute; Departments of [2]Oncology, [3]Pathology, and [4]Medicine, The Sol Goldman Pancreatic Cancer Research Center, The Johns Hopkins Medical Institutions

Through exomic sequencing of a patient with familial pancreatic cancer, we identified a novel pancreatic cancer susceptibility gene, PALB2. We sequenced 20,661 coding-genes in the tumor of a patient with familial pancreatic cancer. Identification of the somatic changes acquired by the patient's cancer also yielded information on the patient's germline DNA. Overall, we observed 15,461 germline variants including 7318 synonymous, 7721 missense, 64 nonsense, 108 splice variants, and 250 small deletions/insertions (54% in-frame). Given that tumors from patients with an inherited tumor suppressor gene often lose the wild-type allele either through loss of heterozygosity or somatic mutation, we examined the patient's tumor and germ-line DNA for genes with evidence of both inherited and somatic variants that were likely to results in the absence of functional protein in the patient's tumor. We observed this patient had a germline 4pb deletion (TTGT at 172 to 175) in PALB2 as well as somatically acquired a transition mutation (C to T) at a canonical splice site (IVS10+2). Germline sequence analysis of 96 additional independent familial pancreatic cancer patients resulted in the identification of three additional truncating mutations in three patients, establishing mutations in PALB2 in addition to its binding partner BRCA2, a known pancreatic cancer susceptibility gene, as the two most common causes of familial pancreatic cancer identified to date. Follow-up studies are underway to better understand the importance of PALB2 in pancreatic cancer in order to improve risk assessment and treatment for this deadly cancer.

380 Detecting Copy Number Variation in Candidate Genes for Colorectal Cancer Risk

Christina Laukaitis, Patricia Thompson

Arizona Cancer Center, University of Arizona, Tucson

Colorectal cancer risk is exaggerated when tumor suppressor genes, such as TP35, MSH2, PTEN, STK11, and APC, are deleted. It is not clear whether the converse is also true—is a person protected from cancer if he/she has additional copies of these genes? One group has shown that people with additional copies of the gene for the detoxifying enzyme Glutathione S-transferase M1 have a lower risk of lung cancer development (Crosbie 2009 Mutat Res). Here, we ask whether there are similar genes affecting colon cancer risk. In order to answer this, we have re-analyzed genotyping data collected from a cohort of individuals undergoing serial colonoscopy as part of two interventional trials. While the intervention showed no change in the risk of future adenoma, genomic DNA was analyzed for single nucleotide polymorphisms (SNPs) in 65 genes involved in a variety of growth factor modulating and DNA repair pathways. In re-analysis, we looked at plots of allele intensity for each individual SNP tested to look for evidence of copy number variation (CNV) in each gene. If two alleles of a two-copy gene are present, plotting the intensity of binding of the test probe for the A gene allele versus the B gene allele for all of the tested samples should give three clusters corresponding to individuals with AA, AB, and BB genotypes. When more or fewer than three clusters are seen, this can mean that duplication or deletion is present at that gene locus. Deletion or duplication has been reported in 48 of the 65 genotyped genes when studied in genome-wide analyses of structural variation. Our curation of the 1536 SNPs analyzed in this candidate gene set has identified 91 SNPs in 35 genes with obvious signatures of gene CNV. We are experimentally confirming these findings using multiplex ligation probe assay (MLPA). We hope to correlate the CNV status of individual genes with the risk of forming a second adenoma to find candidate CNV genes for future studies. This work is limited by the candidate gene approach taken in the original study, the lack of methodology for statistical assignment of risk to copy number variant genes, and knowledge of the rate of gene CNV in average people, and we are developing methodology to overcome those limitations.

381 Functional SNPs in MicroRNAs and Genes Involved in Epithelial to Mesenchymal Transition and Risk for Lung Cancer

Shuguang Leng[1], Amanda Bernauer[1], Christine A. Stidley[2], Maria A. Picchi[1], Elizabeth A. Burki[1], Frank D. Gilliland[3], Richard E. Crowell[4], Timothy Byers[5], **Steven A. Belinsky**[1]

[1]Lung Cancer Program, Lovelace Respiratory Research Institute; [2]Department of Internal Medicine, University of New Mexico; [3]Keck School of Medicine, University of Southern California, Los Angeles; [4]New Mexico VA Health Care System, Albuquerque; [5]Department of Preventive Medicine and Biometrics, University of Colorado Cancer Center, Aurora, CO

Currently, over 700 microRNAs have been identified and are predicted to regulate 20–30% of all human genes, with an average of 200 predicted targets per microRNA. Because each microRNA can regulate hundreds of genes simultaneously, dysfunction of a microRNA could have a major impact in cancer etiology. Polymorphisms in pre-microRNAs (before processing) and microRNA targets can alter processing, expression, and binding to target mRNA to impact carcinogenesis. Recent studies have shown that reduced expression of specific microRNAs may induce the epithelial to mesenchymal transition (EMT), a key developmental program that is activated during cancer invasion and metastasis. A study was initiated to determine whether sequence variations are present in promoter and coding regions of micro-RNAs and the 3'UTR of genes causal for EMT. The genomic region encompassing the promoter region and coding sequence of six microRNAs (has-mir-200c-141 and 200b-200c-429 clusters and has-mir-205) was amplified from DNA isolated from lymphocytes from 20 cases of lung cancer matched to 20 controls by age, gender, and smoking status. The 3'UTR of ZEB1 and ZEB2 and the promoter of E-cadherin were also amplified. Direct sequencing of PCR products identified a total of 32 SNPs and 9 deletions with frequencies > 0.03, 6 of which are newly discovered and 5 are reported but not characterized. The three deletions not in LD with any other SNPs were evaluated for association to lung cancer using the Transgenomics Wave machine for detection. NM lung cancer cases (n = 400) and controls (n = 400) were studied. The 13-base-pair deletion in the E-cadherin promoter was associated with a 2.3-fold (CI, 1.1–4.8, p = 0.02) increased risk for lung cancer in persons smoking < 41 pack years. No association to lung cancer was seen for deletions identified in mir-205 or mir-200c-141. Expectation maximization algorithm was used to construct haplotypes in the promoter regions of the mir-200c and b clusters and E-cadherin. Eighteen Tag-SNPs that capture the major genetic variation across the six regions sequenced have been selected for genotyping using the VeraCode Universal Capture Bead assay in NM cases and controls with the Illumina BeadXpress. We are currently assessing whether identified sequence changes in the promoter regions and 3'UTRs affect transcription of the microRNA and expression of the gene, respectively.

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382 Lung Cancer Chemoprevention With Celecoxib in Ex-Smokers

Jenny T. Mao[1,2], Michael D. Roth[1], Michael C. Fishbein[1], Zuo-Feng. Zhang[1], Jian Yu Rao[1], Denise Aberle[1], Carmack E. Holmes[1], Donald P. Tashkin[1], Lee Goodglick[1], Eva Szabo[3], Steven M. Dubinett[1], Robert Elashoff[1], David Elashoff[1]

[1]University of California, Los Angeles; [2]New Mexico VA Healthcare System and University of New Mexico; [3]National Cancer Institute

Preclinical data suggests that the cyclooxygenase-2 (COX-2)/prostaglandin-E2 (PGE2) pathway plays a pivotal role in carcinogenesis. Overproduction of PGE2 occurs in the setting where COX-2 expression is upregulated and is associated with a variety of carcinogenic mechanisms. Results from a pilot phase IIa trial in high-risk smokers suggested that Celecoxib (a COX-2 inhibitor) might reduce PGE2 production and restore anti-tumor immunity in the lung microenvironment and reduce proliferation indices (Ki-67 labeling index, KI-67 LI) on the bronchial epithelium. These data supported the antineoplastic effect of COX-2 inhibitors and provided the rationale for evaluating their potential in lung cancer chemoprevention. Funded by a U01 mechanism, a phase IIb, randomized, placebo-controlled, crossover pilot study was carried out to determine the feasibility of Celecoxib for lung cancer chemoprevention in ex-heavy smokers (age > 45, > 30 pack years of smoking history and at least one year of smoking cessation). Qualified participants underwent comprehensive screening with low-dose helical CT scan and fluorescence bronchoscopy. Celecoxib (400 mg twice daily) was evaluated for its impact on cellular and molecular events associated with lung carcinogenesis. We prescreened 4,470 subjects, actively screened 323 subjects, performed screening bronchoscopy on 142 subjects, and randomized a total of 137 subjects, of which 101 subjects were evaluable. The primary end point of the study was modulation of the Ki-67 LI on bronchial mucosa following 6 months of treatment. The aggregate mean change in Ki-67 LI for each subject was determined by averaging the change score (pre-treatment versus post-treatment) for all evaluable biopsies from that subject. Modulation of Ki-67 LI in response to treatment was then analyzed by comparing these aggregate mean changes between the treatment versus placebo groups. Primary analysis indicates that 6 months of Celecoxib treatment significantly decreased Ki-67 LI in heavy former smokers by an average of 34%, similar to what was observed in active smokers in the phase IIa study, whereas an average of a 4% increase was observed in the placebo group (p = 0.04; t test). A significant treatment effect based on the interaction between treatment and baseline expressions on Ki-67 LI was also observed using a mixed-effects analysis (p = 0.006). Evaluations of a variety of secondary surrogate endpoint biomarkers are currently underway.

383 Biomarkers of Lung Cancer Risk

York E. Miller, **Fred R. Hirsch**, Wilbur Franklin, Holly Wolf, William Feser, Anna Baron, Robert Keith, Timothy Kennedy, Stephen Belinsky, Marileila Varella-Garcia, Tim Byers.

University of Colorado Cancer Center - Lung Spore

This SPORE has developed and validated biomarkers of lung cancer risk. The focus has been on epithelial cells shed in sputum or from endobronchial biopsies. A high risk cohort of 3,269 subjects with >20 pack-years smoking and airflow obstruction have provided sputum, blood, and urine prospectively for biomarker analysis in nested case control studies. Some 196 incident lung cancers have developed in this cohort. We confirmed that sputum atypia of moderate or worse grade predicts lung cancer within 5 months with a sensitivity of 67% and a specificity of 83%. Sputum atypia is most highly associated with squamous cell lung cancer. In collaboration with Johns Hopkins SPORE, we have validated gene promoter methylation as a risk biomarker. Using a panel of six genes, sensitivity of 64% and specificity of 64% were obtained. The addition of more genes improves the test characteristics. Chromosomal aneusomy in sputum as assessed by a 4-probe FISH panel is even more promising, with a sensitivity of 78% and specificity of 95% within 12 months of diagnosis. Additional FISH probes, selected on the basis of genetic loci frequently increased in copy number in lung cancer, are being evaluated for improvement in test characteristics. We hope to further validate these results using specimens from the NLST. Biomarkers in bronchial epithelial biopsies are being evaluated based on a cohort of 784 high risk individuals who have undergone bronchoscopy, mostly for clinical suspicion of lung cancer. There are 231 prevalent and 22 incident lung cancers in this group. While the overall relationship between endobronchial histology and incident lung cancer is not statistically significant, when the analysis is restricted to squamous cell lung cancer alone (8 cases), the adjusted HR for moderate/severe dysplasia was 2.71 (95% CI: 1.09,6.75). Additional subjects with incident lung cancer are being obtained from collaborators to increase the small numbers on which this analysis is based. In a cross-sectional study, we have determined that the addition of chromosomal aneusomy by FISH to histology results in a stronger relationship to the presence of lung cancer at another site in the patient. The presence of aneusomy in a biopsy resulted in an OR of 4.68 (1.97–11) for all grades of dysplasia and 5.84 (1.37–26) for carcinoma in situ (CIS). The relationship between CIS, chromosomal aneusomy, and invasive cancer is maintained even when those cases of CIS adjacent to an invasive cancer are excluded. These findings support the concept that both chromosomal aneusomy and histologic dysplasia reflect a broader field effect and may be useful in combination as bronchial epithelial biomarkers of risk.

384 TMPRSS2:ERG and SPINK1 in Prostate Cancer Etiology and Progression

Lorelei A. Mucci[1,2,3], Richard Flavin[1,4], Azra Ligon [2,4], Kathryn Penney[1,2,3], Whitney Hendrickson[1,4], Michelangelo Fiorentino[4], Jennifer Stark[1,2,3], Neil Martin[2,4], Blaine Philips[2], Christopher Fiore[4], Jennifer Sinnott[2,3], Jing Ma[1,2], Matthew Freedman[1,4], Stephen Finn[4], Edward Giovannucci[1,2,3], Meir J. Stampfer[1,2,3], Massimo Loda[1,4], Philip W. Kantoff[1,2,4]

[1]Dana Farber/Harvard Cancer Center Prostate Cancer Program, Boston, MA; [2]Brigham and Women's Hospital and Harvard Medical School, Boston MA; [3]Harvard School of Public Health, Boston, MA; [4]Dana Farber Cancer Institute, Boston, MA

ERG and SPINK1 were nominated as top oncogenes in prostate cancer (PCa) through a Cancer Outlier Profile Analysis, an approach that led to discovery of the TMPRSS2:ERG fusion. SPINK1 outlier expression appears as a distinct molecular subset in tumors that lack TMPRSS2:ERG. Some studies suggest both TMPRSS2:ERG- and SPINK1-positive tumors have a more aggressive phenotype. However, not all men who harbor the molecular alterations progress, while some with neither develop disseminated disease, suggesting additional factors are necessary. We are undertaking a comprehensive patho-epidemiology study to explore germline variants, lifestyle factors, or additional markers with TMPRSS2:ERG and SPINK1 in PCa risk and progression. The study is nested among 1,500 men with PCa (1983–2004)\ in the Physicians' Health Study and Health Professionals Follow-up Study. The men were followed for bony metastases and cancer-specific mortality through 2008; 175 have developed lethal disease. We are characterizing TMPRSS2:ERG and SPINK1 on archival tumor tissue specimens. Germline SNPs in genes involved in sex hormone metabolism and IGF/insulin pathways were assayed. Lifestyle information was collected pre- and post-diagnosis. Expression of additional markers was assayed by IHC. The TMPRSS2:ERG prevalence was 42%, 2/3 by deletion. Some 12% of tumors were SPINK1 positive; 1% shared both alterations. The fusion prevalence did not differ by Gleason grade. However, tumors diagnosed at an advanced (64%) versus localized (39%) stage were more likely to be fusion positive. Fusion-positive tumors, particularly by deletion, had substantial reductions in tumor apoptosis (p=0.03). Neither fusion or SPINk1 status predicted lethal outcomes. Fusion-positive tumors showed increased sex hormone signaling, with upregulation of AR (p~0.02) and ER-alpha (p<0.001). On a subset of tumors (N=116) with RNA expression data, an 87-gene signature differentially expressed in fusion-positive and -negative tumors was identified to be related to estrogen signaling. TMPRSS2:ERG tumors may also respond to insulin signaling. Fusion-positive tumors had considerable upregulation of tumor expression of insulin receptor and IGF1R, a finding intriguing since obese men had a lower risk of developing fusion-positive tumors compared to healthy weight (RR=0.32, 95% CI=0.12-0.81). Future work will focus on understanding the role of sex hormones and insulin on prostate cancer progression in combination with the fusion.

385 Energy Balance, Leptin, and Prostate Cancer Risk in the Prostate Cancer Prevention Trial

Marian L. Neuhouser[1], Cathee Till[1], Phyllis Goodman[1], Alan R. Kristal[1] and Michael Pollak[2]

[1]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA; [2]Departments of Medicine and Oncology, McGill University, Montreal, Canada

Overweight and obese men are at increased risk for high-grade prostate cancer. In addition, overweight and obese men have poor prognosis and die of prostate cancer more often than normal weight men. Understanding the biology of energy imbalance in relation to both risk and prognosis is an important translational science topic with bench to bedside applications. For example, lifestyle modifications or effective pharmaceuticals may be part of the treatment portfolio, but in order to devise the best therapies, a better understanding of the obesity-carcinogenesis biology is needed. To this end, we have examined the role played by leptin in relation to prostate cancer and the extent to which leptin may help explain obesity and cancer relationships. Leptin is synthesized by adipose cells as well as other tissues; leptin levels tend to be higher in overweight people due to their overabundance of adipose cells. Leptin has numerous functions including regulation of energy intake via its effect on satiety, modification of insulin sensitivity, and regulation of the immune response and inflammation. Here we examined the association of baseline measures of serum leptin with risk of total, low-grade and high-grade prostate cancer in the Prostate Cancer Prevention Trial (PCPT). Multivariate-adjusted logistic regression tested associations of BMI and serum leptin with total prostate cancer risk and multivariate-adjusted polytomous regression modeled low-grade (Gleason < 7) and high-grade disease (Gleason > 7). We observed inverse associations of obesity with low-grade prostate cancer (OR = 0.78, 95% CI, 0.62–0.96, p, trend =0.02). However, men who were obese (BMI > 30.0) had an increased risk of high-grade prostate cancer (Odds Ratio = 1.36, 95% CI, 1.01–1.82, p, trend = 0.04). High versus low serum leptin was associated with a 25% reduced risk of total prostate cancer (p, trend = 0.01), and risk estimates were similar for low- and high-grade disease. We next examined whether leptin mediated the obesity-cancer associations, even though leptin alone did not appear to be a risk factor. We observed no evidence that leptin mediated the association of obesity with high-grade prostate cancer. We conclude that obesity is a strong risk factor for high-grade prostate cancer in the PCPT, but the biological mechanism is not via leptin. Clinicians should advise men to maintain a healthy weight to reduce prostate cancer risk; further work is needed to understand the biological mechanisms.

386 Acid Exposure Leads to Fibroblast Proliferation and Transformation in Barrett's Esophagus

Ganapathy A. Prasad[1], Navtej S. Buttar[1], Tsung-Teh Wu[2], Lori S. Lutzke[1], Kelly T. Dunagan[1], Lynn S. Borkenhagen[1], Kenneth K. Wang[1]

[1]Gastroenterology and Hepatology and [2]Department of Pathology, Mayo Clinic College of Medicine, Rochester, MN, USA

Background: Chronic acid reflux can cause injury and inflammation, which can activate fibroblasts. Activated fibroblasts are a source of growth factors shown to promote carcinogenesis. We have previously shown the presence of myofibroblasts in BE stroma and the induction of COX-2 expression by in vitro exposure of BE fibroblasts to acid pulses. We aimed to assess the influence of acid exposure on BE fibroblasts in an in vitro environment.

Methods: Primary cultures of BE fibroblasts were derived from pooled biopsies of BE patients without dysplasia. We studied the effect of acid exposure on fibroblast proliferation and apoptosis by exposing fibroblasts in 24 well plates to two 10-minute pulses of acid at varying pHs (4.5, 5.5, and 6.5) along with a control group without acid exposure. Proliferation was assessed by estimating total cell counts using a cell counter (staining with trypan blue to count only viable cells) as well as the MTS assay. Apoptosis rates were calculated by Hoechst staining. We also assessed the transformation of fibroblasts to myofibroblasts using immunocytochemistry, RT-PCR, and Western Blot analysis for for alpha smooth muscle [ASMA] expression using standard methods in fibroblasts exposed to two acid pulses of 10-minute duration , 1 hour apart over 5 days.

Results: The median fibroblast cell count in wells exposed to acid (90,000) was significantly greater than the count in wells not exposed to acid (52,000) (p= 0.009). This was also confirmed by the MTS assay where the median absorbance in fibroblasts exposed to acid (1.75) was significantly greater than that in cells not exposed (1.1) (p=0.04). The rate of apoptosis was not different between fibroblasts exposed (0.06) and not exposed to acid (0.08) (p=0.72). Immunohistochemistry, RT-PCR, and western blotting revealed consistent evidence of fibroblast transformation to myofibroblasts as demonstrated by the significantly higher expression of ASMA in cells exposed to acid pulses compared to controls. Scanning electron microscopy also confirmed the change in the morphology of fibroblasts after exposure to acid and the appearance of cytoplasmic microfilament rich structures in cells expressing ASMA.

Conclusions: Exposure to acid leads to stromal fibroblast proliferation and transformation to myofibroblasts in BE fibroblast cultures. The role of acid exposure, myofibroblasts, and myofibroblast-derived growth factors in the progression of neoplasia in BE needs further study.

387 Novel Proteomic Signature of Risk for Lung Cancer

S.M. Jamshedur Rahman[1], Adriana L. Gonzalez[2], Erin H. Seeley[3], Lisa J. Zimmerman[4], Xueqiong J. Zhang[5], Ming Li[5], Takefumi Kikuchi[6], Pierre Chaurand[3], M. Lisa Manier[3], Sandy Olson[2], Ronak N. Shah[1], Alison Miller[1,7], Joe B. Putnam[8], York E. Miller[10], Wilbur A. Franklin[11], David P. Carbone[6], Yu Shyr[5], Richard M. Caprioli[3], **Pierre P. Massion**[1,7,9]

[1]Division of Pulmonary and Critical Care Medicine, [2]Department of Pathology, [3]Mass Spectrometry Research Center, Departments of [4]Biochemistry and [5]Biostatistics, [6]Division of Hematology and Oncology, Vanderbilt-Ingram Comprehensive Cancer Center; [7]Veterans Affairs Medical Center, Nashville; Departments of [8]Thoracic Surgery and [9]Cancer Biology, Vanderbilt-Ingram Comprehensive Cancer Center; [10]Pulmonary Division, Denver Veterans Affairs Medical Center; [11]Department of Pathology, University of Colorado, Denver

A biomarker signature to identify at-risk individuals who will progress to lung cancer would be of great value. Airway histology alone does not address this risk in a sufficient manner. Our hypothesis is that a tissue proteomic signature specific to a subgroup of preinvasive lesions associated with a high risk for lung cancer may serve as an important tool for the design of early detection and chemoprevention strategies. In this study, we selected 11 MALDI mass spectrometry (MS) features (m/z values) based on a statistical ranking from previous studies, and we tested these features in an independent validation dataset consisting of 69 tissue specimens from 60 patients to test their ability to distinguish normal bronchial epithelium and low-grade preinvasive lesions from high-grade preinvasive and invasive lung tumors. The prediction accuracy was 79%, with 73% sensitivity and 83% specificity. We identified 10 of the 11 candidate features corresponding to seven proteins, thymosin β4, ubiquitin, acyl-CoA binding protein (ACBP), cytochrome C, cystatin A, S100A11, and macrophage migration inhibitory factor. Each of these proteins was validated by immunohistochemistry, western blotting, and MALDI imaging mass spectrometry (IMS). MALDI IMS localized the candidate biomarker proteins specifically to high-grade preinvasive lesions and invasive cancer tissues. Further validation of this novel proteomic signature in a prospective cohort of patients may facilitate selection and monitoring of high risk individuals for lung cancer screening and chemoprevention trials.

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388 Racial Survival Disparity in Head and Neck Cancer Results From Low Prevalence of Human Papillomavirus Infection in Black Oropharyngeal Cancer Patients

Kathleen Settle[1], Marshall R. Posner[2], Lisa M. Schumaker[1], Ming Tan[1], Mohan Suntharaligam[1], Olga Goloubeva[1], Scott E. Strome[1], Robert I. Haddad[2], Shital S. Patel[1], Earl V. Cambell, III[1], Nicholas Sarlis[3], Jochen Lorch[2], **Kevin J. Cullen**[1]

[1]University of Maryland Marlene and Stewart Greenebaum Cancer Center, Baltimore, MD; [2]Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA; [3]Sanofi-Aventis, Bridgewater, NJ

The burden of squamous cell carcinoma of the head and neck (SCCHN) is greater for blacks than for whites, especially in oropharyngeal cases. We previously showed retrospectively that disease-free survival was significantly greater in white than in black SCCHN patients treated with chemoradiation, the greatest difference occurring in the oropharyngeal subgroup. Oropharyngeal cancer is increasing in incidence and in its association with humanpapillomavirus (HPV) infection; HPV-positive oropharyngeal cancer patients have significantly better outcomes (versus HPV-negative). These collective data led to the present analyses of overall survival (OS) in our retrospective cohort and of OS and HPV status (tested prospectively in pretreatment biopsy specimens) in the phase 3, multicenter TAX 324 trial of induction chemotherapy followed by concurrent chemoradiation in SCCHN patients. Median OS in the retrospective cohort of 106 white and 95 black SCCHN patients was 52.1 months (white) versus only 23.7 months (black; $P = 0.009$), due entirely to OS in the subgroup of patients with oropharyngeal cancer—69.4 months (whites) versus 25.2 months (blacks; $P = 0.0006$); no significant difference by race occurred in survival of non-oropharyngeal SCCHN ($P = 0.58$). In TAX 324, 196 white patients and 28 black patients could be assessed for HPV status. Median OS was significantly worse for black patients (20.9 months) than for white patients (70.6 months; $P = 0.03$) and dramatically improved in HPV-positive (not reached) versus HPV-negative (26.6 months, 5.1 hazard ratio) oropharyngeal patients ($P < 0.0001$), 49% of whom were HPV-16 positive. Overall, HPV positivity was 34% in white versus 4% in black patients ($P = 0.0004$). Survival was similar for black and white HPV-negative patients ($P = 0.56$). This is the first prospective assessment of confirmed HPV status in black versus white SCCHN patients. Worse OS for black SCCHN patients was driven by oropharyngeal cancer outcomes, and that for black oropharyngeal cancer patients by a lower prevalence of HPV infection. These findings have important implications for the etiology, prevention, prognosis, and treatment of SCCHN.

389 Genetic Variants in UGT2B Genes and Breast Cancer Risk

Chang Sun[1], Catherine Southard[1], David B. Witonsky[1], Olufunmilayo I. Olopade[1,2], **Anna Di Rienzo**[1]

Departments of [1]Human Genetics and [2]Medicine, University of Chicago, Chicago, IL

Breast cancer risk is influenced by the balance between estrogen and androgen levels. Since UDP-glucuronosyltransferase 2 family, polypeptide B cluster (UGT2B) plays an important role in the clearance of steroid hormones and of multiple carcinogens, it has been proposed that UGT2B genetic variation contributes to breast cancer risk. However, due to the high sequence similarity among UGT2B genes, sequence and expression variation in this family have not been thoroughly investigated, including in recent genome-wide expression and association studies. We resequenced the coding regions, promoters, conserved non-coding regions, and putative liver-enriched transcription factor binding sites (50.8 kb in total) within UGT2B cluster in 56 unrelated HapMap individuals (24 YRI, 22 CEU, and 10 ASN). Our resequencing survey identified 439 SNPs, most of which were not present in dbSNP. Together with the EGP resequencing (http://egp.gs.washington.edu/directory.html) and HapMap genotype data, our data yielded 195 tagging SNPs for genotyping. We then isolated RNA and DNA from 81 normal breast and 31 liver samples and measured UGT2B gene expression by real-time PCR. The tagging SNPs are now being genotyped by Sequenom iPLEX in these samples. The correlation between them will be evaluated by linear regression to identify variation affecting UGT2B expression levels.

As part of our efforts to characterize expression variation, we also found that the UGT2B7 gene is not expressed in breast, contrary to what was previously claimed (due to improper RT-PCR and immunoblot assay design). Also, allelic imbalance was observed in the UGT2B15 gene in liver ($P<0.001$) but not in breast ($P=0.06$), which indicated that one or more cis-regulatory SNPs could influence UGT2B15 expression liver. Further resequencing of the UGT2B15 promoter region identified seven SNPs (-506A/T, -508A/G, -818G/T, -1139C/T, -1397C/A, -1579G/A, and -1844T/C) in LD with the allelic imbalance SNP. By reporter gene promoter assay, functional SNPs were identified to be -818G/T and -1139C/T. By chromatin immunoprecipitation (ChIP), -818 was verified to be within a transcription factor, Nrf2 (nuclear factor erythroid derived 2-like 2) binding site in both HepG2 and MCF-7 cells.

390 Variants in the CDKN2B and RTEL1 Regions Are Associated With High-Grade Glioma Susceptibility

Margaret Wrensch[1], Robert B. Jenkins[2], Jeffrey S. Chang[1], Ru-Fang Yeh[1], Yuanyuan Xiao[1], Karla V. Ballman[2], Mitchel Berger[1], Jan C. Buckner[2], Susan Chang[1], Paul A. Decker[2], Caterina Giannini[2], Chandralekha Halder[2], Thomas M. Kollmeyer[2], Matthew L. Kosel[2], Daniel H. LaChance[2], Lucie McCoy[1], Brian Patrick O'Neill[2], Joe Patoka[1], Alexander R. Pico[1], Michael Prados[1], Charles Quesenberry[3], Terri Rice[1], Amanda L. Rynearson[2], Ivan Smirnov[1], Tarik Tihan[1], Joe Wiemels[1], Ping Yang[2], John K. Wiencke[2]

[1]University of California, San Francisco; [2]Mayo Clinic; [3]Kaiser Permanente

The causes of glioblastoma and other devastating gliomas remain obscure. To discover new candidate genes that might influence glioma susceptibility, we conducted a principal component-adjusted genome-wide association study (GWAS) of 275,895 autosomal variants among 692 adult high-grade glioma patients (622 from the San Francisco Adult Glioma Study (AGS) and 70 from the Cancer Genome Atlas (TCGA) versus 3,992 controls (602 from AGS and 3,390 from Illumina iControlDB [iControls]). For replication, we then analyzed the 13 SNPs with $p<10–6$ using completely independent data from 176 high-grade glioma patients versus 174 controls from the Mayo Clinic. Rs1412829 in chromosome 9p21 (near CDKN2B) had discovery p-value 3.4*10–8, replication p-value 0.0038, and combined p-value 1.85*10-10. Rs6010620 intronic to RTEL1 had discovery p-value 1.5*10-7, replication p-value 0.00035, and combined p-value 3.40*10-9. For both SNPs, the direction of association was the same in discovery and replication phases.

391 Genetic Variation in the Prostate Stem Cell Antigen (PSCA) Gene Confers Susceptibility to Urinary Bladder Cancer: A Genome-Wide Association Study

Xifeng Wu[1], Yuanqing Ye[1], Lambertus A. Kiemeney[2], Patrick Sulem[3], Thorunn Rafnar[3], Giuseppe Matullo[4,5], Daniela Seminara[6], Teruhiko Yoshida[7], Norihisa Saeki[7], Angeline S. Andrew[8], Colin P. Dinney[1], Bogdan Czerniak[1], Zuo-feng Zhang[9], Anne E. Kiltie[10], D. Timothy Bishop[10], Paolo Vineis[5,11], Stefano Porru[12], Frank Buntinx[13,14], Eliane Kellen[15,16], Maurice P. Zeegers[14,16], Rajiv Kumar[17], Peter Rudnai[18], Eugene Gurzau[19], Kvetoslava Koppova[20], Jose Ignacio Mayordomo[21], Manuel Sanchez[21], Berta Saez[22], Annika Lindblom[23], Petra de Verdier[23], Gunnar Steineck[24], Gordon B. Mills[1], Alan Schned[8], Shen-Chih Chang[9], Jie Lin[1], David W. Chang[1], Katherine S. Hale[1], Tadeusz Majewski[1], H. Barton Grossman[1], Steinunn Thorlacius[3], Unnur Thorsteinsdottir[3], Katja KH Aben[2], J. Alfred Witjes[2], Kari Stefansson[3], Christopher I. Amos[1], Margaret R. Karagas[8], Jian Gu[1]

[1]The University of Texas M. D. Anderson Cancer Center; [2]Radboud University Nijmegen Medical Centre, Netherlands; [3]deCODE Genetics, Iceland; [4]University of Torino, Italy; [5]Institute for Scientific Interchange Foundation, Italy; [6]U.S. National Cancer Institute; [7]National Center Research Institute, Japan; [8]Dartmouth Medical School; and 16 other institutions

Purpose: Genome-wide association study (GWAS) is an efficient way to identify genetic susceptibility for complex disease. To identify novel bladder cancer susceptibility loci, we conducted a GWAS in Caucasians and replicated results in three U.S. studies and nine European populations. **Methods:** We carried out a primary screen by analyzing 556,429 haplotype tagging SNPs in 969 histologically-confirmed bladder cancer cases and 957 age- and gender-matched controls; for replication, we first evaluated the top 50 SNPs (p<10E-4) and the top 10 SNPs in 8q24 (p< 5x10E-3), a region contains cancer susceptibility loci for several cancers, in three additional U.S. populations of 1,713 cases and 3,871 controls. We then validated the top candidate in nine European populations with additional 3,985 cases and 34,762 controls. **Results:** One SNP on chromosome 8q24 (rs2294008) showed consistent significant association with bladder cancer across the discovery set (p= 7.34x10E-4) and the three U.S. replication sets (p= 3.53×10-5). This SNP also exhibited a significant association with bladder cancer in European populations (p=9.83x10E-5). Combining all the populations in this study (6,667 cases and 39,590 controls), the overall p-value for rs2294008 with bladder cancer was 2.14x10E-10, and the allelic OR was 1.15 (95% CI: 1.10–1.20). Rs2294008 is a missense variation that alters the start codon and causes the truncation of 9-amino acids of the prostate stem cell antigen (PSCA). In vitro reporter gene assay showed that the variant allele significantly reduced the promoter activity of PSCA gene in three bladder cancer cell lines. Resequencing of the PSCA genomic region of Caucasians found that the SNP is the only missense SNP with high frequency, and all the high-frequency SNPs are in strong linkage disequilibrium. **Conclusions:** Based on population evidence, genomic resequencing, and biochemical data, rs2294008 in the PSCA gene is likely a causal variant that contributes to an increased risk of bladder cancer. Future studies are needed to decipher the physiological role of PSCA and rs2294008 in vivo and the biological mechanisms for their involvement in bladder cancer predisposition.

392 Relationship Between Germ-Line Copy Number Variations and Sun Exposure/Host Factors in Melanoma-Prone Families Without Known Mutations

Xiaohong Rose Yang, Margaret Tucker, Alisa Goldstein

Division of Cancer Epidemiology & Genetics, National Cancer Institute

Genomic copy number variations (CNVs) have recently been recognized as significant sources of genetic variation that may contribute to disease susceptibility. We previously observed that number of copy number gains and number of genes located within CNVs were significantly higher among cutaneous malignant melanoma (CMM) cases compared to unaffected individuals in melanoma-prone families without known mutations. The goal of this study was to examine the association between germline CNVs and major risk factors for melanoma including sun exposure, pigmentation, nevi, and MC1R variables in these melanoma-prone families. We conducted a genome-wide search for CNVs using the Nimblegen 385K whole-genome array-CGH. We analyzed blood-derived genomic DNA from 78 individuals (61 CMM cases and 17 spouses) selected from 30 melanoma-prone families without known mutations. We used the Nexus Copy Number™ built-in Rank Segmentation algorithm to identify significant CNVs (significance threshold=0.000001; minimal number of probes per segment=5; log2 ratio=0.2 for low gain, 0.5 for high gain, -0.3 for loss, -0.7 for homozygous loss). We used a T-test and linear regression to test the associations between CNV variables and melanoma risk factors (dysplastic nevi, number of nevi, hair color, eye color, skin color, freckles, solar injury, response to sun exposure, and MC1R variants). We found that, compared to individuals with no or few freckles (mean number of copy number gains/individual, 3.2±2.9), individuals with freckles tended to have more copy number gains (6.2±3.9, P=0.07, moderate freckles; 8.1±5.7, P=0.002, many freckles). Individuals with solar injury also had increased number of copy number gains (4.3±3.7, absent/mild; 6.4±5.2, P=0.11, moderate; 7.8±5.2, P=0.03, severe). We did not find significant associations between any CNVs and the other risk factors evaluated. These results suggest a dose-dependent relationship between freckles and solar injury and germline copy number gains, implying that freckles and solar injury may increase melanoma risk through altered genomic rearrangement. We will further identify the unique CNVs that are associated with these exposures and search for candidate genes and pathways that are influenced by these CNVs.

393 Understanding and Eliminating Oncogenic EGFR Signaling in Malignant Gliomas

Jaime Acquaviva, Abraham Boskovitz, **Al Charest**

Department of Neurosurgery and Molecular Oncology Research Institute, Tufts Medical Center, Tufts University School of Medicine, Boston, MA

Background: Malignant gliomas account for the majority of primary brain cancers and are considered one of the most insidious malignancies in humans. Our research focuses on the use of animal models of glioblastoma multiforme (GBM) to understand the molecular and genetic contributors to disease initiation, maintenance, and resistance to therapies. The epidermal growth factor receptor (EGFR) signaling pathway plays a crucial role in GBM pathogenesis: initiating the early stages of tumor development, sustaining tumor growth, promoting infiltration, and mediating resistance to therapy. The importance of this pathway is highlighted in the fact that EGFR is mutationally activated in >50% of GBM tumors.

Methods: Consistent with this observation, we have created a genetically engineered conditional EGFR mouse model of GBM and established that EGFR overexpression in adult mice promotes the development of GBMs. We hypothesized that specific gene mutations will confer resistance to targeted therapeutic agents and, by eliminating the responsible gene(s), we can sensitize tumors to those drugs.

Results: Using cells established from these GBM tumors, we demonstrated that sensitivity to tyrosine kinase inhibitors (TKI) is dictated by the genetic makeup of the tumors. We performed cell growth and apoptosis assays on these cells and demonstrated, for example, that Iressa treatment of EGFR-positive and PTEN-null tumor cells results in cytostaticity, whereas treatment of EGFR positive tumor cells is a cytotoxic effect. To better understand these differences at the molecular lever, we studied these results in the context of global phospho-proteomic analysis. We performed phospho-tyrosine proteomic scans using mass spectrometry on treated versus non-treated cells and established system network pathways that relate to the observed behaviors of the cells.

Conclusions: Lack of PTEN expression directs EGFR signaling networks towards pathways that are no longer responsive to TKI action. In deciphering signaling components of TKI resistance, we strive to identify and overcome key members that are responsible for drug treatment resistance.

394 Integrative Epigenomic Approach to Biomarker Discovery During Esophageal Carcinogenesis

Hector Alvarez[1], Joanna Opalinska[2], Li Zhou[2], Yongkai Mo(2), Elizabeth Montgomery[1], Jean Wang[1], John Greally[2], Anirban Maitra[1], **Amit Verma**[2]

[1]Johns Hopkins School of Medicine; [2]Albert Einstein Cancer Center, Bronx, NY

The advent of global profiling technologies provides an unprecedented opportunity to integrate large-scale datasets across independent platforms, such that one can not only catalog molecular alterations in human cancer, but also elucidate the underlying mechanisms for these changes within a given sample. We used a novel epigenomic platform combining global DNA methylation profiling, DNA copy number alteration analysis (array CGH), and gene expression profiling to study stepwise progression of normal squamous epithelium to dysplasia and cancer using paired sets of primary patient samples. Here, we provide an integrated approach for comparing large-scale genomic, epigenetic, and transcriptomic datasets, using tissue obtained from patients undergoing endoscopic mucosal biopsy for Barrett esophagus. By integrating datasets obtained from multistep morphological progression within the same individual, we are able to create a comprehensive model of genetic progression for Barrett esophagus, and in many instances, pinpoint the precise mechanism(s) for the observed anomaly. On a panoramic scale, we find that epigenetic alterations are widespread at the earliest stages in multistep carcinogenesis and are characterized by global hypomethylation in diseased tissue. In contrast, gene-specific hypermethylation, often accompanied by hemi-allelic deletions, is observed at individual tumor suppressor loci like CDKN2B/p15, leading to their transcriptional silencing in Barrett esophagus. Integrative analysis also reveals that promoter hypomethylation and/or copy number aberrations are the basis for many of the significantly upregulated transcripts, such as GATA6 or DMBT1, observed during multistep progression. The validation of these upregulated targets by single-gene assays in a larger panel of primary mucosal biopsy samples, including their association with adverse prognosis in EACs, as well as functional studies to confirm their oncogenic properties in EAC cell lines, validates our integrative approach for identifying new molecular biomarkers of disease progression and for elucidating their mechanisms of disruption in a comprehensive and unbiased manner.

395 MicroRNA Biomarkers for Ovarian Cancer

John R. Chevillet[1], Rachael K. Parkin[1], Patrick S. Mitchell[1], Nicole Urban[2], Muneesh Tewari[1]

[1]Division of Human Biology; [2]Division of Public Health Sciences and Pacific Ovarian Cancer Research Consortium/Specialized Program of Research Excellence, Fred Hutchinson Cancer Research Center

MicroRNAs (miRNAs) are a class of small (approximately 22 nt long) regulatory RNA molecules that play important roles during normal development and homeostasis and are often dysregulated in cancer. The Tewari laboratory has recently shown that (i) tumor-derived miRNAs are released in a cell-free form into the circulation where they exist in a highly stable state and can be measured quantitatively using qRT-PCR and (ii) in a proof-of-principle study examining a small pilot group of candidate miRNAs, serum miRNA markers could distinguish healthy individuals from patients with metastatic prostate cancer. These results suggest that miRNAs represent a novel class of blood-based biomarkers for cancer. Ovarian cancer is the most lethal of the gynecologic malignancies and the fifth most common cause of death in women overall. Most patients are diagnosed at a late stage and with a poor prognosis. In addition, ovarian cancer is a clinically heterogeneous disease, with varying degrees of resectability and subtype-specific responses to treatment. We are investigating whether tumor-derived miRNAs circulating in the blood or present in ascites fluid may represent a source of minimally-invasive biomarkers for the early detection, prognosis, and personalized treatment of ovarian cancer.

396 Leptin Deficient Mice Develop Prostate Epithelial Hyperplasia

Jennifer A. Doll[1], Susan M. Wcislak[1], Philip Fitchev[1], Mona Cornwell[1], Chung Lee[2], Charles B. Brendler[1], Paul Lindholm[3], Susan E. Crawford[1]

[1]Department of Surgery, NorthShore University HealthSystem, Evanston, IL; Departments of [2]Urology and [3]Pathology, Northwestern University, Chicago, IL

Obesity is a major health epidemic in the United States and significantly increases the risk of developing a wide spectrum of diseases including prostate cancer (PCa). Large studies have shown a consistent association between obesity and an increased risk of dying from PCa. Obese individuals have an increase in size and number of mature adipocytes that function as an endocrine organ, secreting many soluble proteins, called adipokines, including leptin. Circulating levels of leptin are elevated in obese individuals. Leptin can also induce angiogenesis and stimulate proliferation of androgen-insensitive PCa cells, and serum levels are elevated in PCa patients with aggressive disease. These data suggest that leptin may contribute to PCa progression; therefore, we investigated the prostate phenotype in ob/ob mice that become obese but are leptin-deficient.

We harvested prostate and surrounding adipose (peri-prostatic) tissues from 3–12 month old ob/ob mice (n=20). Tissue was formalin-fixed, paraffin-embedded, and H&E stained. Histology was compared to age-matched wild-type C57Bl/6 prostate tissue (n=25), the background strain of the ob/ob mice. Adipose tissue and the ventral, lateral, and dorsal prostates were examined. As expected, there is significantly more peri-prostatic adipose tissue in the ob/ob mice with an increased number and larger adipocytes as compared to wild-type. In addition, there were fewer multilocular adipocytes in the ob/ob mice. Within the prostate, the ventral and dorsal prostate of the ob/ob mice demonstrated significant epithelial hyperplasia compared to wild-type, which has a predominantly single layer of epithelial cells lining the glands. The lateral prostate was only mildly affected, with occasional tufts of epithelial proliferation. In the ventral prostate, in addition to the epithelial proliferation, there was an accumulation of vacuoles within the epithelial cells, with some cells containing multilocular vacuoles. In both the ventral and dorsal prostate, the epithelial proliferation was accompanied by cellular and nuclear atypia. In addition, dilated intra-epithelial blood vessels were common in the dorsal prostate in ob/ob mice, a feature rarely seen in the normal mouse prostate. These data suggest that adipokines may drive prostate hyperplasia and promote PCa progression.

397 A New Preclinical Platform for Pancreas Cancer

Sunil R. Hingorani

Clinical Research and Public Health Sciences Divisions, Fred Hutchinson Cancer Research Center, and University of Washington School of Medicine, and Seattle Cancer Care Alliance, Seattle, WA

Enormous advances in the biomedical sciences have raised tenable hope of a new era of personalized medicine. Significant hurdles to realizing this goal include the inordinately high and evolving degree of genetic and biologic diversity in carcinomas, even within categories of defined organ and cell type. In addition, the tissue culture and xenograft preclinical models systems that have been instrumental in elucidating fundamental principles of cancer biology do not recapitulate the essential geometry or tissue and stromal interactions encountered in the native disease. As a result, the ability to kill cancer cells in these contexts has had limited impact on treating the diseases in patients. We have attempted to address these challenges with respect to pancreatic ductal adenocarcinoma (PDA), or pancreas cancer, a highly aggressive and almost uniformly fatal malignancy. Anatomy and biology conspire not only to elude early detection but also to hinder scientific inquiry of pancreas cancer. To circumvent these constraints, we have developed state-of-the-art genetically engineered mouse models that faithfully recapitulate—from inception to invasion—the multiple distinct genetic and histopathologic routes to pancreas cancer seen in the human disease. These animal models also appear to encompass the biological diversity and complexity seen in patients. The models have helped elucidate and substantiate basic principles of pancreas cancer pathogenesis including defining the initiating event, establishing pancreatic intraepithelial neoplasias (PanINs) as bona fide precursors to invasive disease, revealing the existence of unique genetic trajectories to PDA, and the discovery that the sequence as well as the total complement of mutations determines the clinical and biological behavior of the resultant disease. We have also used these model systems to identify critical aspects of the robust stromal response that appear to promote tumorigenesis while also limiting the ability of therapeutic agents to penetrate the tumor. In addition to revealing important aspects of disease pathobiology, these model systems now also form the basis of a new preclinical platform, a Murine Clinical Trials Program, to aid the rigorous development, evaluation, and, ultimately, selection of early detection, chemoprevention, and therapeutic strategies most likely to impact patients in the clinic. Examples of the use of this platform to advance these strategies will be presented.

398 Human Proteome Arrays for Auto-Antibody Identification in Clinical Cancer Studies

John Kenten, Jessica Suschak, Kris Roth, Bandele Jeffery-Coker, Fei Yin, Eli N. Glezer

Meso Scale Diagnostics, Gaithersburg, MD

Early detection, identification, and classification of cancers still present considerable challenges. Innovations in several areas of research have provided novel and powerful approaches with which to tackle these challenges. Autoantibodies to tumor antigens have been found in cancer patients. Such autoantibodies are potentially ideal diagnostic biomarkers, as they are present in serum, are stable, and can be measured using established serological techniques. In order to discover new autoantibody responses in cancer patients, we have developed an approach based on the generation of protein arrays for systematic identification of auto-reactive antibodies. Our approach makes use of several key technologies: sequence-curated clone libraries, in vitro protein expression, and a sensitive detection technology based on a proprietary combination of electrochemiluminescence detection and patterned arrays (Multi-Array® Technology, Meso Scale Diagnostics). Using this combination of technologies, we plan to generate immobilized arrays of >1,000 proteins, with a high density of proteins per well in a 96-well microtiter plate format. The array of human proteins is selected from our collection of 60,000 mammalian clones (characterized by sequencing). The protein arrays will be used to screen cancer patient sera and controls.

During the initial development of this platform, we optimized the protocols for in vitro protein expression and labeling, specifically for immobilization on our Multi-Array plates. With this approach, we have demonstrated detection of antigen-specific antibodies down to a concentration of 0.1 ng/mL. We have also validated this approach in a model system, with the detection of auto-reactive antibodies in the sera from 29 patients with a range of auto-immune diseases (Sjogren's syndrome, mixed connective tissue disease, scleroderma, systemic lupus erythematosus, and primary bilary cirrhosis), using nine cloned autoimmune antigens. In parallel, we also optimized approaches for spotting in vitro expressed proteins in high density spot/well arrays. In these studies, we evaluated a selection of electrode surfaces and protein coupling methods in combination with various spotting methods and demonstrated serological measurements in a high density spot/well array.

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399 AIDS Lymphoma as a Model System for Studying the Role of Macrophages in the Pathogenesis of Cancer

Michael S. McGrath[1], Leanne C. Huysentruyt[1], Susanna L. Lamers[2], Marco Salemi[3], Sylvia Silver[1], Amy Chadburn[4], Debra L. Garcia[5], Leona W. Ayers[1]

[1]AIDS and Cancer Specimen Resource (ACSR); [2]BioInfoExperts, Thibodaux, LA; [3]Department of Pathology, Immunology and Laboratory Medicine, University of Florida; [4]Feinberg School of Medicine, Northwestern University, [5]AIDS and Cancer Specimen Resource Central Operations and Data Coordinating Center (ACSR CODCC), San Francisco, CA

Background: The incidence of AIDS related lymphoma (ARL) has decreased since the institution of highly active anti-retroviral therapy (HAART); however, HIV infected individuals still develop lymphoma at a rate 10X above background. Preliminary reports suggested that a subset of ARLs contained HIV infected macrophages (MO) that may contribute to ARL pathogenesis, similar to MO-driven AIDS dementia,. The goal of this research is to: (1) identify ARL characteristics that are associated with HIV infected MOs and 2) test whether HIV infected MOs influence the pathogenesis of ARL. **Methods:** Tissue microscopic arrays (TMA) containing ~150 cases of non-CNS ARLs (from 1982–2007) and frozen multisite autopsy specimens from patients who died with ARL were obtained through the AIDS and Cancer Specimen Resource (ACSR) and analyzed for MO CD68 and HIV p24 expression. The EBV status of the TMA ARLs was previously determined by the ACSR. Two multisite autopsy cases containing HIV+ MOs were studied in greater detail by extracting HIV DNA from tumor-associated MOs (TAM) and performing sequence evolution analysis. As MOs represent a long-lived HIV reservoir cell population, the HIV sequence evolution rates were used as migration markers for metastatic sites of tumor containing the TAMs. **Results:** The ARLs had variable levels of TAMs as identified by CD68 staining ranging from 2 to 47%. HIV p24 was present in TAMs, many times in perivascular regions similar to the pattern seen in AIDS dementia. There was a highly significant ($p<0.001$) increase in the frequency of HIV+ ARL cases post-HAART (after 1997; 49%) as compared to pre-HAART (before 1997; 26%), although the rate of EBV infection with the ARLs remained the same (44% vs. 40%). Multisite mapping of HIV evolution within ARLs yielded the following information: (1) ARL-specific HIV sequences were present in all tumor sites but were not present in non-tumor sites and (2) the rate and pattern of HIV evolution appeared to parallel tumor metastasis within the patients in vivo. **Conclusion:** More than one-half of ARLs contain HIV+ TAMs, independent from the presence of EBV. ARL HIV+ TAMs represent a novel long-term infected reservoir of a potentially disease-specific HIV. Additionally, HIV DNA provides a molecular marker capable of tracking spread of TAMs and, thus, tumor in vivo. These data may have important implications for studying the role of TAMs in the pathogenesis of cancer metastasis and as targets for MO-specific therapeutic development.

400 Multiplexing the BioCD for Cancer Diagnostics and Prognostics

David D. Nolte[1], Patrick O'Neil[1], Guillermo Garcia-Manero[2]

[1]Purdue University; [2]The University of Texas M. D. Anderson Cancer Center

The BioCD is a versatile immunoassay optical microarray that operates either in high-throughput multiplexed mode or in real-time binding mode. The optical detection is primarily interferometric and label-free but also has hybrid modality that enables fluorescence detection and sandwich assays. The high throughput mode uses laser scanning spinning-disc interferometry (SDI) and scans thousands of antibody spots in minutes. The real-time binding mode uses molecular interferometric imaging (MI2) to monitor immunoassay binding with high imaging resolution for accurate measurements of kinetic rates. For each of these detection modes, the assay format uses a thin oxide on silicon to establish a common-path interferometric quadrature condition. The antibody immobilization is to the silica surface, using butylaldehyde or isocyanate chemistries. The BioCD has potential for high-throughput multiplexing, and in this NCI-funded project, we are exploring its application to cancer diagnostics and prognostics.

The principal focus of the prognostic research is acute lymphocytic leukemia (ALL). Three biomarkers show modified expression in subsets of ALL that correlates with prognosis. For instance, the downregulated expression of one or more of the markers p15, p57, and p73 in bone marrow tissues correlates with poor prognosis, while unaltered expression of these markers correlates with positive response to chemotherapy. We have established standard curves for these markers and are poised to assay up to 60 biological specimens for which tissue array results are available. As part of the system validation, we have also investigated the BioCD applicability to prostate cancer (PCa) and epithelial ovarian cancer (EOC). Standard curves for PSA in serum have been established down to 1 ng/ml, and PSA has been quantified in three prostate cancer patient sera using the BioCD. Standard curves for the ovarian markers CA125, HE4 and osteopontin have been established, and CA125 has been detected in an ovarian cancer patient serum. These initial results in the complex serum protein background are among some of the first demonstrations of interferometric molecular marker detection in serum.

401 Identification of Fused Transcripts in Colon Cancers as Biomarkers

Nickolas Papadopoulos, HeeJung Hwang

The Ludwig Center at The Johns Hopkins Medical Institutes, Baltimore, MD

Genetic alterations can unequivocally distinguish cancer from normal cells in an individual. Characterization of specific genomic aberrations in colorectal cancer from TCGA and other genome-wide sequencing studies has led to the successful identification of a number of genes involved in cancer development. However, cancer genes contributing to oncogenesis can arise as a result of somatic rearrangements that result either in fusion transcripts (e.g., BCR–ABL1) or in transcriptional deregulation (e.g., Ig-MYC). Although the large majority of the known somatically rearranged genes associated with cancer are found in leukemias, lymphomas, and sarcomas, the recent discoveries of an EML4-ALK fusion in non-small-cell lung cancers and ETS fusion genes in prostate cancers demonstrate that recurrent translocations in solid tumors may be more frequent than previously thought. Furthermore, such alterations create a unique sequence, devoid from similar background sequences, specific to the cancer cells that carry them. Thus, identification of such fusion transcripts can both point to potential targets for the development of therapies and cancer specific diagnostic tools. To identify fused expressed transcripts in colon cancer, we created paired-end libraries for massively parallel sequencing from cDNA from eight colorectal cell lines. For each sample, we sequenced on average 23 million paired-end tags, resulting in a 20x coverage of the transcriptome. We identified both intra- and inter-chromosomal rearrangements. Fusion transcripts were validated by RT-PCR from the sample that the sequencing tags were derived. We have cataloged all the translocations, deletions, and inversions identified in these colon cancer cell lines. The prevalence of the fusion-transcripts is being tested in a large number of colorectal and other tumors. Currently, the utility of such genetic alterations as biomarkers is being explored.

402 Development of a Novel Human Xenograft Model for the Study of Skin Cancer Biology In Vivo [WITHDRAWN]

Yosef Refaeli[1], Antonio Jimeno[2], Xiao-Jing Wang[3], Dennis Roop[1], Marianne Berwick[4], David Norris[1]

Departments of [1]Dermatology, [2]Medicine, [3]Pathology, Anschutz Medical Campus, University of Colorado Denver, Aurora, CO; [4]University of New Mexico, Division of Epidemiology and CTRC, Albuquerque, NM

Although the direct patient tumor xenograft model (DPTXM) is a powerful tool, host animals are immunocompromised, limiting the utility of this system for studying interactions between tumors and the immune system. Humanized chimaeric mice transplanted with human fetal liver HSCs or cord blood HSCs have been developed to address this issue. However, at least 106 HSCs are required for engraftment, and reconstitution is still a rare event. Also, since primary HSCs need to be freshly implanted and additional HSCs cannot usually be obtained from the same source, the results from one cohort of mice are restricted to that experiment. Finally, because transplanted human lymphoid cells remain restricted to the thymus, bone marrow, and spleen, this system cannot be used to study tumor-immune system interactions. By transplanting human conditionally transformed long-term HSCs (ctlt-HSC) into sublethally irradiated immunodeficient mice, we have developed a novel chimaeric mouse with a human hematopoietic system. Our novel approach provides several advantages over older systems. First, because we use a conditionally immortalized HSC cell line as the source, variability is reduced, facilitating comparisons across experimental conditions. Second, we can begin the process with far fewer cells than current approaches because we will generate HSC cell lines for xenotransplantation. Third, our humanized chimaeric mice produce mature human T and B cells that can infiltrate into tumors via blood vessels and lymphatic vessels; hence, these animals can be used to study tumor-immune system interactions. The goal of Project 2 is to establish a tumor xenograft model in chimaeric NOD/SCID/ß2M–/– mice reconstituted with human ctlt-HSCs. This system will be used to evaluate CSC properties, niche, and CSC-host interactions and to test therapeutic strategies.

We use two strategies to develop humanized xenochimaeric, tumor-bearing mice. First, we will obtain both tumor and blood samples from an individual patient. We will conditionally immortalize HSCs isolated from peripheral blood, then transplant them into sublethally irradiated NOD/SCID/ß2M–/– mice. Meanwhile, tumors will be passaged by xenografting in nude mice. Once NOD/SCID/ß2M–/– have been reconstituted with ctlt-HSC, we will then transplant tumors derived from the same patient into these animals. In parallel, we will also develop a system in which we use a universal donor ctlt-HSC line and induce T cell tolerance to an allogeneic (non-self) tumor. These unique systems will allow us to study tumor-immune system interactions in vivo.

403 Novel Approaches for the Molecular Profiling of Clinical Specimens

Michael A. Tangrea, Michael R. Emmert-Buck

Laboratory of Pathology and Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD

The Pathogenetics Unit (PGU) develops novel approaches to investigate the molecular pathology of cancer with an emphasis on prostate and esophageal studies. These new methods are subsequently applied to gene and biomarker discovery projects, often as collaborative, multidisciplinary efforts. Currently, we are working on the development of two new technologies: expression microdissection (xMD) and layered expression scanning (LES). Based on immuno-targeting, xMD converts microdissection from an operator-dependent to an operator-independent mode, thus eliminating user bias and the need to laboriously procure cells. Through the NIH Director's Challenge Award and a cross-discipline team of scientists, we are further improving the resolution of xMD to obtain pure populations of subcellular structures for proteomic analysis. When fully developed, xMD may offer several advantages over current microdissection methods, including greater speed and precision. Layered expression scanning (LES) is a new technology co-developed by the Pathogenetics Unit and 20/20 GeneSystems, Inc., through a Cooperative Research and Development Agreement (CRADA). The method utilizes a layered array of membranes for high-throughput molecular analysis and can be applied to a variety of life science platforms, including tissue specimen and electrophoretic gels. Overall, we anticipate that xMD and LES will become complementary tools that will assist the PGU and other investigators in phenotype- and expression-based profiling studies of cell populations in tissue sections.

404 "Next Gen" Diagnostic and Prognostic Metagenes for High Risk Childhood Cancer

Timothy Triche[1], Jonathan Buckley[1], Elai Davicioni[2], Jim Anderson[3], Phil Kapranov[4]

[1]University of Southern California; [2]University of British Columbia; [3] University of Nebraska; [4]Helicos, Inc.

RNA and DNA based biomarker profiles are increasingly used to improve diagnosis, predict prognosis, and select therapy. Most are derived from complex data sets based on microarray gene expression data, but many other forms of genomic data, like unannotated RNA expression, SNPs, Copy Number, Loss of Heterozygosity, Methylation, and Proteomic data are becoming available as well. Further, "next gen" sequencing data for both DNA sequence and RNA digital gene expression are appearing. The challenge now is to incorporate these data into linked databases of clinical and genomic data in order to derive next-generation biomarker profiles. We are utilizing RNA and DNA microarray data from our UO1 SPECS project on high-risk childhood sarcomas from Children's Oncology Group clinical trials in conjunction with "next gen" DNA sequencing and non-biased cDNA (total RNA) expression data coupled with clinical protocol data, to create very dense, comprehensive views of the high risk childhood cancer genome in order to derive improved biomarker profiles that can be used to better predict molecular diagnosis, prognosis, and potential therapeutic targets. Examples of these diagnostic metagenes, composed of selected genomic features linked to diagnosis, treatment, and outcome data, will be presented. A comparative analysis of multiple platforms for RNA and DNA data will also be discussed, particularly in relationship to informative genomic regions of interest.

405 Identification of EGFR-Src-Stat3 Complex Using a Novel Microchannel Protein Complex Detection System

Ying-Nai Wang[1], Hirohito Yamaguchi[1], Chao-Kai Chou[1], Nan Jing[2], Jun Kameoka[2], **Mien-Chie Hung**[1]

[1]Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX; [2]Department of Electrical and Computer Engineering, Texas A&M University, College Station, TX

Analysis of protein-protein interaction is indispensable for current molecular biology research, but existing biochemical methods cannot provide us single molecular level information of multiple protein complexes. To overcome the disadvantage, we have recently developed a new lab-on-chip platform and methodology that can quickly and quantitatively detect three proteins interactions in limited amounts of cell lysate samples. Using this system, single molecules can be analyzed based on their fluorescent profile, and their profiles are plotted into 2-dimensional time-coincident photon burst diagrams (2DTP).

Epidermal growth factor receptor (EGFR) is frequently overexpressed or mutated in a variety of human cancers, and EGFR signaling plays an important role in tumor progression including cancer cell proliferation, survival, and metastasis. EGFR triggers various critical oncogenesis pathways by interacting with different proteins such as Src, Stat3, Her2, c-Met, Grb2, etc. Thus, using our novel microchannel system, we identified the dynamics of EGFR-Src, EGFR-Stat3, and EGFR-Src-Stat3 complex before and after EGF stimulation. Importantly, we demonstrated that this system requires only a small amount of samples and relatively short processing time. Together, the microchannel protein detection system may be a promising tool for future molecular cancer research as well as clinical application for cancer diagnosis.

406 Integrated Analysis of Genetic and Epigenetic Alterations in Lethal Metastatic Prostate Cancer

Srinivasan Yegnasubramanian[1], Julia Engelmann[1], Wennuan Liu[2], Martin Aryee[1], William G. Nelson[1], Jianfeng Xu[2], Rafael Irizarry[1], G. Steven Bova[1], William B. Isaacs[1]

[1]Johns Hopkins University; [2]Wake Forest University

Cancer cells appear to establish their malignant phenotype through the acquisition of numerous somatic genetic and epigenetic genome alterations. Recent evidence suggests that such genetic and epigenetic alterations may cooperate to drive cancer initiation and progression. We have developed a novel technology platform for highly integrative, parallel analysis of genome-wide copy number and DNA methylation alterations. Applying this technology to multiple distinct prostate cancer metastatic deposits and matched normal tissues from each of 14 subjects from a rapid autopsy program, we observe that both copy number and DNA methylation alterations are clonally maintained during metastatic dissemination. Furthermore, this analysis revealed several genomic loci that exhibit bi-allelic inactivation of putative tumor suppressor regions through a cooperation of copy number loss and DNA methylation in a recurrent fashion. These analyses will help us to better understand the cooperation between genetic and epigenetic processes during carcinogenesis and disease progression and may also help to identify novel targets for prostate cancer treatment and diagnosis.

407 Role of Mst1 and Mst2 in Hepatacellular Carcinoma Pathogenesis

Dawang Zhou[1], Claudius Conrad[1], Ji-Sun Park[1], Fan Xia[1], Bernhard Payer[1], Yi Yin[1], Gregory Y. Lauwers[1], Wolfgang Thasler[2], Jeannie T. Lee[1], Joseph Avruch[1], **Nabeel Bardeesy**[1]

[1]Massachusetts General Hospital; [2]LM University, Munich

The elucidation of the molecular pathogenesis of hepatocellular carcinoma (HCC) is needed to inform improved therapeutic approaches to the disease. We have recently identified the Mst1/2 ser/thr kinases as important HCC tumor suppressors. Mst1/2 are the orthologs of the Drosophila tumor suppressor Hippo, which promotes apoptosis and inhibits cell proliferation during development. Hippo action is mediated by a set of critical substrates that are conserved in humans and that, in turn, suppress the transcriptional coactivator, Yorkie, whose human ortholog, Yap, is an established oncogene and regulator of organ size. By generating Mst1/2 KO mice, we found that Mst1 and Mst2 are required to maintain quiescence of liver cells and that their acute ablation results in Yap activation, massive liver overgrowth, resistance to apoptosis, and the development of HCC within 6–8 weeks. Significantly, we have determined that ~30% of human HCCs have deregulation of the Mst1/2-Yap signaling axis. These results establish Mst1/2 as important tumor suppressors relevant to the pathogenesis of HCC in humans and point to Yap as a critical downstream target.

408 High-Grade Astrocytoma-Specific Molecular Targeting

Waldemar Debinski[1], Hetal Pandya[1], Steve Kridel[2], Denise Gibo[1]

[1]Brain Tumor Center of Excellence and [2]Department of Cancer Biology, Wake Forest University School of Medicine

There has been only a modest progress in implementing effective means of treatment for patients with glioblastoma multiforme (GBM) during the last decades. We have previously uncovered a plasma membrane receptor that is over-expressed in around 80% of patients with GBM and not normal brain, interleukin 13 receptor alpha 2 (IL-13Rα2). This receptor has served the development of targeted cytotoxins, viruses and vaccines, which have already entered the clinic and demonstrated efficacy. IL-13Rα2 remains an attractive target for other molecular means to diagnose, image and treat GBM. We have recently generated three different agents that recognize IL-13Rα2 through various ways that have potential to be further developed for imaging and treatment of the disease. First, a heptapeptide was selected from the disulphide-constraint peptide phage display library that binds specifically IL-13Rα2. This peptide has two important features: (i) it binds the receptor independent of the native ligand, and (ii) it induces receptor's internalization in preliminary experiments. Thus, the peptide can potentially serve as an imaging agent for the effectiveness of anti-GBM treatments. Interestingly, the linear form of the peptide binds the receptor more effectively than the disulphide-constraint. Second, a doubly-targeted agent was designed and produced, which is composed of receptor-recognition unit (IL-13K, a mutated form of native ligand), a portion of a bacterial toxin that enables cytosolic transport (D2) and a nuclear localization signal (NLS); IL-13.E13K.D2.NLS. This recombinant protein recognizes cells over-expressing IL-13Rα2 and according to its design's purpose, delivers the C-terminal portion to the cells' nuclei. This opens a novel way of delivering drugs/labels not only to a targeted subpopulation of cells, but also directly to their preferable/desirable site of cellular action. Third, six novel monoclonal antibodies (MAb) were generated that recognize specifically IL-13Rα2 in both humans and dogs. Dogs develop spontaneous malignant gliomas and their clinical, pathological and molecular features, including over-expression of IL-13Rα2, resemble human disease. Also, recent clinical studies with MAb in brain tumors demonstrated efficacy and the availability of several of them against an attractive target in GBM will allow a careful examination of this not well-explored approach in the treatment of GBM. We are continuously examining potential clinical application of various targeted peptides/proteins/antibodies as anti-GBM drugs and/or imaging agents.

409 Timed Sequential Treatment With Cyclophosphamide, Doxorubicin, and an Allogeneic Granulocyte-Macrophage Colony-Stimulating Factor-Secreting Breast Tumor Vaccine: A Chemotherapy Dose-Ranging Factorial

Leisha A. Emens[1,2,3], Justin M. Asquith[1,2], James M. Leatherman[1,2], Barry J. Kobrin[1,2], Silvia Petrik[1,2], Marina Laiko[1,2], Joy Levi[1,2], Maithili Daphtary[1,2], Barbara Biedrzycki[1,2], Antonio C. Wolff[1,2], Vered Stearns[1,2], Mary L. Disis[4], Xiaobu Ye[1,2], Steven Piantadosi[1,2,5], John H. Fetting[1,2], Nancy E. Davidson[1,2], Elizabeth M. Jaffee[1,2,6,7,8,9]

[1]Sidney Kimmel Comprehensive Cancer Center, [2]Department of Oncology, [3]Pathobiology Program, Johns Hopkins University School of Medicine, Baltimore, MD; [4]The Tumor Vaccine Group of the University of Washington, Seattle, WA; [5]University of Washington, Seattle, WA, and the Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA; Departments of [6]Pathology and [7]Pharmacology, and Programs in [8]Immunology and [9]Cellular and Molecular Medicine at the Johns Hopkins University School of Medicine, Baltimore, MD

Purpose: Granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting tumor vaccines have demonstrated bioactivity, but may be limited by disease burdens and immune tolerance. We tested the hypothesis that Cyclophosphamide (CY) and Doxorubicin (DOX) can enhance vaccine-induced immunity in breast cancer patients. **Patients and Methods:** We conducted a 3-by-3 factorial (response surface) dose-ranging study of CY, DOX and a HER-2+, allogeneic, GM-CSF-secreting tumor vaccine in 28 patients with metastatic breast cancer. Patients received 3 monthly immunizations, with a boost 6–8 months from study entry. Primary objectives included safety and determination of the chemotherapy doses that maximize HER-2-specific immunity. **Results:** Twenty-eight patients received at least 1 immunization; 16 patients received 4 vaccinations. No dose limiting toxicities were observed. HER-2-specific delayed type hypersensitivity developed in most patients who received vaccine alone or with 200 mg/m2 CY; HER-2-specific antibody responses were enhanced by 200 mg/m2 CY and 35 mg/m2 DOX; higher CY doses suppressed immunity. Analyses revealed CY and DOX doses of 200 and 35 mg/m2 respectively as the combination that produced the highest antibody responses. **Conclusions:** First, immuno-therapy with an allogeneic, HER-2+, GM-CSF-secreting breast tumor vaccine alone or with CY and DOX is safe, and induces HER-2-specific immunity in metastatic breast cancer patients. Second, the positive immunomodulatory activity of low dose CY has a narrow therapeutic window, with an optimal dose not exceeding 200 mg/m2. Third, factorial designs provide an opportunity to identify the most active combination of interacting drugs in patients. Further investigation of the impact of chemotherapy on vaccine-induced immunity is warranted. Trial Registration: clinicaltrials.gov identifier: NCT00093834.

410 Metabolic Engineering of Cell Surface Sialic Acids for Selective Immunotherapy of Cancer

Zhongwu Guo

Department of Chemistry, Wayne State University, Detroit, MI

Tumor-associated carbohydrate antigens (TACAs) expressed on tumor cell surfaces, many of which are sialooligosaccharides, are important molecular targets or templates in the development of cancer vaccines or cancer immunotherapies. However, among scores of TACAs identified thus far, only a few are useful, and most TACAs have the immunotolerance problem, namely that the protein conjugates of these TACAs fail to provoke immune responses useful for cancer immunotherapy. To overcome this problem and to make use of the tolerated but richly expressed TACAs for the treatment of cancer, a novel immunotherapeutic strategy based on metabolic engineering of sialo-TACAs on cancer cells has been developed. First, a sialo-TACA derivative that contains an unnatural sialic acid residue is synthesized and utilized as the vaccine to establish a specific immune response. Next, the correspondingly modified mannosamine derivative is used as the biosynthetic precursor of the unnatural sialic acid to initiate the expression of the sialo-TACA derivative on cancer cells. Subsequently, the provoked immune system will target and eradicate the glycoengineered cancer. Our immunological studies demonstrated that the N-phenylacetyl derivatives of GM3 (GM3NPhAc) and sTn (sTnNPhAc) could form effective vaccines to provoke strong and specific immune responses, and studies on many mannosamine derivatives showed that N-phenylacetyl mannosamine (ManNPhAc) was an excellent precursor for glycoengineering of a number of tumor cell lines. Antibodies obtained with vaccines made of GM3NPhAc mediated highly selective cytotoxicity to ManNPhAc-treated cancer cells in the presence of ManNPhAc-treated normal cells or untreated tumor cells. Moreover, vaccination using GM3NPhAc conjugates in combination with administration of ManNPhAc was proved to be effective for the treatment of metastatic tumors in vivo.

411 Inhibition of Tumorigenesis and Immune Suppression With Inhibitors of the STAT-3 Pathway

Amy B. Heimberger[1], Ling-Yuan Kong[1], Jun Wei[1], S. Farzana Hussain[1], Mohamed Abou-Ghazal[1], Arup Chakraborty[1], Izabela Fokt[1], Wei Qiao[1], Wei Sun[1], Gary E. Archer, Jr.[2], John H. Sampson[2], Gregory N. Fuller[1], Elizabeth A. Grimm[1], Howard Colman[1], Raymond Sawaya[1], Frederick F. Lang[1], Waldemar Priebe[1]

[1]The University of Texas M. D. Anderson Cancer Center; [2]Duke University Medical Center

The signal transducer and activator of transcription (STAT)-3 pathway is expressed across a wide variety of malignancies, including central nervous system (CNS) gliomas and melanoma. Phosphorylation of STAT-3 (p-STAT-3) drives the fundamental components of tumorigenesis and a primary mediator of immune suppression. P-STAT-3 is induced in a variety of immune subsets upon encountering the tumor environment or the secreted products from the tumor. Specifically, p-STAT-3 induces FoxP3 in T regulatory cells (Tregs), down modulates co-stimulatory molecules and pro-inflammatory cytokines in antigen presenting cells, and induces immunosuppressive CNS microglia/macrophages. Cancer stem cells (CSCs) isolated from human CNS gliomas, which recapitulate many of the features of human gliomas and are mediators of chemo and radiation resistance, produce a variety of immune suppressive products such as macrophage inhibitory cytokine 1 (MIC-1) and express CTLA-4 and B7H1 that trigger T cell apoptosis, inhibit T cell proliferation and function, and induce Tregs. The STAT-3 pathway is overactive in CSCs and treatment with either p-STAT-3 inhibitors, STAT-3 siRNA or differentiation reverses the CSC induction of T cell apoptosis and Tregs, and restores T cell effector functions. Furthermore, the p-STAT-3 inhibitors reverse the immune suppressive phenotype of human microglia by reducing MIC-1 production. Thus, STAT-3 inhibitors could be utilized to modulate the redundant immunosuppressive mechanisms in cancer patients and could be used as chemotherapy modulators or radiation sensitizers.

We have developed orally administered small molecule inhibitors (WP1066, WP1193) of the p-STAT-3 pathway with excellent CNS penetration and with a favorable toxicity profile. Long-term survival was observed in 80% of mice with established intracerebral syngeneic melanoma (B16) treated with WP1066 ($p<0.05$) and also markedly enhanced the in vivo efficacy of IFN-alpha. Although WP1066 did not induce immunological memory or enhance humoral responses, WP1066 reduced the production of immunosuppressive cytokines and chemokines, inhibited Tregs, and increased the cytotoxic immune responses of T cells. This in vivo therapeutic efficacy is ablated in nude model systems and with in vivo depletions of CD8+ effector T cells. Thus, the p-STAT-3 inhibitors may have utility in combination with other immunotherapy and vaccine approaches. Our intention is to bring the p-STAT-3 inhibitors into phase I/II clinical trials within the next 18 months for patients with CNS melanoma metastasis that are typically excluded from clinical trials and in patients with primary gliomas.

412 Chemotherapy Can Enhance the Therapeutic Potential of Vaccine-Mediated Immunotherapy

James Hodge[1], Sofia Gameiro[1], Jorge Caballero[1], Jack Higgins[1], Amanda Boehm[1], Alex Franzusoff[2], Jeffrey Schlom[1]

[1]Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD; [2]GlobeImmune, Inc., Louisville, CO

Purpose: Previous studies have shown that chemotherapy given prior to vaccine can inhibit vaccine mediated antitumor immunity. Since chemotherapy is standard of care for many cancer types, the possibility that chemotherapy can be used concomitantly with vaccine was evaluated. We hypothesized that one could take advantage of the fact that certain chemotherapy regimens induce transient pancytopenia, which is followed by a recovery phase. We examined if administering vaccine during the T-cell recovery phase would enhance the effectiveness of the vaccine. Moreover, we examined the effects of chemotherapy on the quantity and function of regulatory T cells. Many non-small cell lung cancer (NSCLC) patients undergo surgery followed by standard-of-care adjuvant chemotherapy, which includes cisplatin in combination with vinorelbine. In spite of therapy, the median survival of patients with metastatic disease is less than 10 months. **Experimental Design:** We evaluated the potential for biological synergism between the standard-of-care chemotherapy regimen and a recombinant yeast-CEA vaccine in a mouse model of NSCLC. We also examined the translational potential of our preclinical findings by evaluating the effect of chemotherapy on CTL-mediated cytolysis of human NSCLC tumor cell lines in vitro. **Results:** These studies demonstrate for the first time that (a) the combination of cisplatin plus vinorelbine modulates CD4+, CD8+, CD19+, natural killer, and regulatory T-cell populations in healthy mice; and (b) cisplatin plus vinorelbine combined with heat-killed recombinant yeast-CEA vaccine (i) is superior to either modality alone at reducing tumor burden and (ii) increases vaccine mediated antigen-specific T-cell responses. Moreover, cisplatin plus vinorelbine modulates the cell surface expression of immunologically relevant molecules and improves antigen-specific CTL mediate cytotoxicity in vitro.

Conclusions: These finding suggest potential clinical benefit for the combined use of recombinant yeast vaccine and cisplatin-based chemotherapy regimens.

413 Understanding IL-12 Tachyphylaxis: IFN-Gamma as the Master Regulator of Post-IL-12 T-Suppressor Cell Homeostasis in the Tumor Microenvironment

Mehmet O. Kilinc*, Tao Gu*, Rachael B. Turner, Jamie L. Harden, Lauren P. Virtuoso, **Nejat K. Egilmez** (*equal contributors)

Department of Microbiology and Immunology, School of Medicine and Biomedical Sciences, University at Buffalo

Sustained intratumoral delivery of IL-12 reverses tumor-mediated immune suppression via rapid activation of tumor-resident CD8+ T-effector/memory cells (Tem) and a concurrent elimination of CD4+ CD25+ Foxp3+ T-suppressor cells from tumors. However, this reversal is transient and is followed by a rebound in intratumoral T-suppressor cells to levels exceeding pre-therapy intensity within 7 days. Repeated stimulation results in the intensification of the T-suppressor cell rebound and a diminished ability to restore Tem activity. Recent analysis of the mechanisms underlying treatment–induced T-suppressor cell elimination and rebound demonstrated a central role for IL-12-induced IFN-gamma in both these processes. IL-12-mediated elimination of T-suppressor cells from tumors was dependent on tumor-associated CD8+ Tem and required IFN-gamma. Further studies demonstrated that CD8+ Tem eliminated T-suppressors directly via FasL-mediated apoptosis and that autocrine IFN-gamma was critical to upregulation of FasL on CD8+ Tem. These events occurred within 72 hours of treatment. Longer-term monitoring of tumors and tumor-draining lymph nodes (TDLN) revealed that induction of IFN-gamma was followed by a 5-fold increase in indoleamine 2, 3 dioxygenase (IDO) mRNA in tumors and the TDLN. IDO expression required IFN-gamma as treatment failed to induce IDO in IFN-gamma knockout (GKO) mice. IDO production was followed by T-suppressor cell expansion in the TDLN between days 3 to 7. Administration of D-1-methyl tryptophan (D1-MT), an inhibitor of IDO activity, resulted in a delay in T-suppressor cell expansion and extended CD8+ Tem activity, establishing a link between the IFN-gamma-IDO axis and post-therapy T-suppressor cell rebound. Co-administration of D1-MT and IL-12 to tumor-bearing mice resulted in complete tumor regression in 45% of the mice whereas neither reagent alone achieved complete regression. These data further delineate the molecular mechanisms underlying the dichotomous role of IFN-gamma in immune activation and regulation. Importantly, our findings also demonstrate that selective blocking of IFN-gamma-driven feedback inhibition without impacting its pro-inflammatory activity can dramatically enhance the antitumor efficacy of IL-12 therapy. Clinical implications of these findings are discussed.

414 Molecularly Targeted Chemotherapy Using a Novel EphA2 Immunoconjugate

Jeong-Won Lee[1,2], Hee Dong Han[1], Mian M.K. Shahzad[1,3], Seung Wook Kim[1], Lingegowda S. Mangala[1], Alpa M. Nick[1], Chunhua Lu[1], Rosemarie Schmandt[1], Hye-Sun Kim[1,4], Shenlan Mao[5], John Gooya[5], Christine Fazenbaker[5], Dowdy Jackson[5], Charles N. Landen Jr.[1], Robert L. Coleman[1], **Anil K. Sood**[1]

[1]University of Texas M. D. Anderson Cancer Center; [2]Samsung Medical Center, Seoul, Korea; [3]Baylor College of Medicine; [4]Cheil General Hospital and Women's Healthcare Center, Seoul, Korea; [5]Medimmune, Inc., Gaithersburg, MD

Background: EphA2 is overexpressed in many human cancers, but is either absent or present at low levels in normal epithelial tissues. Based on this differential expression, we utilized a novel EphA2-targeted immunoconjugate (1C1-mcMMAF) for molecularly targeted chemotherapy delivery in ovarian carcinoma.

Methods: Antibody binding and internalization assays were performed to test for specificity of 1C1-mcMMAF. In vitro (cell viability and apoptosis) and in vivo (orthotopic animal models of ovarian cancer) assays were carried out to determine the therapeutic efficacy of 1C1-mcMMAF.

Results: Antibody binding and internalization assays confirmed the specificity of 1C1-mcMMAF for EphA2 positive cells. Moreover, in vitro assays demonstrated loss of ovarian cancer cell viability in an EphA2-specific manner following treatment with this immunoconjugate. In vivo treatment with 1C1-mcMMAF resulted in 85 to 98% growth inhibition ($P < 0.001$) in the orthotopic HeyA8 and SKOV3ip1 models. Compared to controls, the EphA2 immunoconjugate was also effective in the chemotherapy-resistant HeyA8-MDR model ($P = 0.01$). Even in bulkier disease models, the EphA2 immunoconjugate was highly effective in causing regression of established tumors, resulting in longer survival. The anti-tumor effects of the therapy were related to decreased proliferation and increased apoptosis of tumor and associated endothelial cells.

Conclusion: These results indicate that the EphA2 targeted immunoconjugate provides therapeutic benefit in preclinical models of ovarian carcinoma and support further clinical development.

415 Phase I Immunotherapy in Women with Metastatic Breast Cancer Using Anti-CD3 x Anti-Her2/neu Bispecific Antibody Targeted T Cells

Lawrence G. Lum[1], Zaid Al-Kadhimi[1], Ritesh Rathore[2], Pamela Davol[2], Cassara Skuba[3],Qin Liu[4], Francis Cummings[2], Joseph P. Uberti[1], Voravit Ratanatharathorn[1]

[1]Karmanos Cancer Institute and Wayne State University, Detroit; [2]Roger Williams Medical Center, Providence; [3]Michigan State University, Lansing; [4]University of Massachusetts

Women with stage IV metastatic breast cancer have limited treatment options since toxicities from chemotherapy and radiotherapy become limiting. Nontoxic immunotherapy approaches to improve targeting and cytotoxicity directed at breast cancer are needed. Our earlier study showed that anti-CD3 activated T cells (ATC) could be expanded in culture and then armed with anti-CD3 x anti-Her2/neu bispecific antibody (HER2Bi). The armed ATC mediate enhanced specific cytotoxicity, proliferate, and induce cytokine/chemokine secretion. In a Phase I trial using ATC armed with Her2Bi, 19 Stage IV BrCa patients (pts) were treated with 8 infusions (twice/week) for 4 weeks totaling 40 (6 pts), 80 (2 pts), 160 (7 pts), and 320(1 pt) billion ATC armed with Her2Bi without dose-limiting toxicities. The most frequent side effects were chills, fever, and hypotension that were easily controlled with medications. BrCa patients had decreases in CEA, CA 27-29, and serum Her2/neu receptors. One pt had partial response with a decrease in her liver metastatic lesions. None of the pts developed human anti-mouse antibody responses. PBMC depleted of armed ATC continue to exhibit cytotoxicity directed at SK-BR-3 cells. These provide strong evidence that endogenous immune cells developed and persisted up to 4 months after treatment. Increasing proportions and absolute numbers of CD25+ cells in CD4+ and CD8+ subsets were observed as a function of treatment with nearly all CD4+ and CD8+ cells being CD25+ by 1 week post-final infusion. Significant treatment-associated elevations (several log increases) of circulating IFNγ, TNFα, IL-2, IL-5, IL-10, IL-12p70, and IL-13 were detected in serum of patients beginning 2 weeks after initiation of infusions. There was a 3 log increase of mean (n=9) serum IL-12p70 from 0 to 1200 pg/ml. Infusions induced a Th1 polarization. Results from the phase I clinical trial suggest that Her2Bi-armed ATC activate the endogeneous immune system to generate an adaptive immune responses despite the presence of high tumor burdens. Overall survival for 19 women (All) treated on the phase I protocol with the median survival for the HER2/neu 3+ group was 56 months, 21 months for the Her2 0–2+ group, and 26 months for the entire group. The median survival for the 9 pts with Her2/neu negative disease was 21.5 months. Together these data are encouraging and strongly suggest infusions of targeted T cells may immunized the patient against their own tumor antigens. This strategy may lead to the development and persistence of long-term cytotoxic T lymphocytes that improve overall survival.

416 Anti-TGF-Beta as a Cancer Therapeutic in Mice and Humans: A Phase I Study of GC1008, a Human Anti-TGF-Beta Monoclonal Antibody in Patients With Advanced Malignant Melanoma or Renal Cell Carcinoma

John C. Morris[1], Lauren Wood[1], Masaki Terabe[1], Geoffrey I. Shapiro[2], Antoinette R. Tan[3], Donald Lawrence[2], Thomas Olencki[4], Bruce J. Dezube[2], Michael Reiss[3], Frank J. Hsu[5], **Jay A. Berzofsky**[1]

[1]National Cancer Institute; [2]Dana-Farber Harvard Cancer Center; [3]Cancer Institute of New Jersey; [4]Ohio State University; [5]Genzyme Corporation

TGF-beta is a broadly active cytokine that can suppress early growth of tumors, but at later stages it is often overexpressed and promotes tumor growth and suppresses anti-tumor immunity. Besides the tumor itself, TGF-beta is made by certain immune cells such as type II NKT cell-activated myeloid cells and Foxp3+ T regulatory cells, and is involved in negative regulation of immunity. In preclinical studies in mice, we found that a mouse monoclonal antibody 1D11 blocking all isoforms of TGF-beta could prevent tumor recurrence in one model and reduce metastases in another tumor model. We have now translated this strategy into a phase I clinical trial of GC1008, a human IgG4 monoclonal anti-TGF-beta antibody that neutralizes all three TGF-beta isoforms. We reported at ASCO (June, 2008) initial results of the first-in-cancer multicenter phase I trial of GC1008 safety and effectiveness as a single agent in malignant melanoma and renal cell cancer. Patients who had failed at least one prior therapy were treated in cohorts of 3–6 patients per dose in a dose escalating study of 0.1, 0.3, 1, 3, 10, and 15 mg/kg of IV GC1008 at weeks 0, 4, 6, 8, with pharmacokinetic studies after the first dose and multiple doses. As of April 21 2008, the data demonstrated that no dose limiting toxicities were noted up to 15 mg/kg. Nine of 22 patients reported serious adverse events unlikely to be related to treatment (due to underlying disease), but other events possibly related to treatment, all grade 1 or 2, included skin rash (including 2 cases of eruptive keratoacanthomas), fatigue, headache, epistaxis, gingival bleeding, and GI symptoms. There was one well-differentiated squamous cell cancer of the skin in a patient with a history of this cancer. Five patients showed biological effects on their tumor, including one partial remission of 89% not progressing for over 52 weeks after initial GC1008 treatment, three mixed responses with some tumors regressing while others progressed, and one case of stable disease. Phase II studies are being planned in other cancers of GC1008 as a single agent and in combination with other cancer therapeutics.

417 Induction of Anti-Vil-6 Specific Responses as a Novel Strategy to Control Kshv-Like Disease in the Siv/Rrv-Infected Rhesus Macaque

Beata U. Orzechowska[1,2], Ryan D. Estep[1,2], Megan O'Connor[1,2], Michael K. Axthelm[1,2,3], **Scott W. Wong**[1,2,3,4]

[1]Vaccine and Gene Therapy Institute and [2]Department of Molecular Microbiology and Immunology, Oregon Health & Science University; [3]Division of Pathobiology and Immunology, Oregon National Primate Research Center; [4]Knight Cancer Institute, Oregon Health & Science University

Rhesus macaque rhadinovirus (RRV) is closely related to Kaposi's sarcoma-associated herpesvirus (KSHV), the etiological agent associated with Kaposi's sarcoma, primary effusion lymphoma, multicentric Castleman's disease and some non-Hodgkin's lymphomas (NHL). Rhesus macaques (RM) co-infected with SIVmac239 (SIV) and/or RRV17577 (RRV) develops lymphoproliferative disease (LPD) that can manifest as extra-nodal NHL. Plasma samples from these animal exhibited significant interleukin-6 (IL-6)-like activity, which we have found to be derived from the viral IL-6 open reading frame (ORF). In addition, immunohistochemical analysis of tissue sections revealed that RRV vIL-6 is abundantly expressed throughout the lesions. To define if RRV vIL-6 is involved in the development of RRV-associated disease, two strategies were developed. First, a recombinant vIL-6-Fc fusion protein was created and utilized to vaccinate two RM (Cohort 1) followed by two subsequent boosts two months apart (Phase 1). Both vIL-6-Fc vaccinated RM developed robust anti-RRV vIL-6 antibody responses. In Phase 2, both RM, including 4 non-vaccinated RM (Cohort 2), were experimentally inoculated with SIV to induce immunodeficiency. At 8 weeks post SIV infection, all animals were subsequently infected with RRV (Phase 3). At 68 weeks post RRV-infection, both cohorts of animals were subsequently re-infected with RRV and monitored for RRV-associated disease. Our second strategy involved the creation of a vIL-6 knockout (RRV vIL-6ko) utilizing our infectious and pathogenic RRV-BAC. Here, two RM (Cohort 3) were experimentally inoculated with SIV and 8 weeks later with the RRV vIL-6ko virus and monitored for RRV-associated disease. Cohort 2 has developed RRV associated disease, whereas Cohorts 1 and 3 exhibit no evidence of disease for over three years. Results from both approaches indicate that RRV vIL-6 is absolutely required for persistent lymphadenopathy or LPD.

418 Molecular Targeted Combinational Chemopreventive Drug Development: Promise and Progress for the Prevention and Treatment of Colorectal Cancer

Chinthalapally V. Rao[1], Ronald Lubet[2], Levy Kopelovich[2], Vernon E. Steele[2]

[1]Department of Medicine, Hem-Onc Section, University of Oklahoma Cancer Institute, Oklahoma University Health Science Center, Oklahoma City, OK; [2]Chemopreventive Agent Development Research Group, Division of Cancer Prevention, National Cancer Institute

Clinical and preclinical studies suggest that molecular targeted small molecule drug development approaches very promising at the preclinical and clinical level for the prevention and treatment of several epithelial cancers. NSAIDs such as aspirin, sulindac, and celecoxib, a cyclooxygenase (COX)-2 inhibitor; DFMO, an ODC inhibitor, atorvastatin, a HMG-R inhibitor, Targretin, a RXR-modulator and giftnib, a EGFR inhibitors have been shown to reduce the risk of colon and other epithelial cancers. However, their prolonged administration at higher doses add significant efficacy but cause for a concern for unwanted toxicity. For example, celecoxib is highly effective in the prevention of colonic polyps but its prolonged administration at higher doses increase cardiovascular risk, a major concern. Thus, developing different molecular targeted chemopreventive combinations may provide additive and synergistic efficacy at low doses, without any unwanted side effects. Using preclinical models of colon cancer we have identified several promising combinational molecular targets and generated impressive data using both single agents and multiple agents. These targets include 5-Lipoxygenase (5-LOX)/COX-2; COX-2 and HMG-R; ODC and COX; and p53 and COX-2; and RXR and ER-beta. Thus, development of agents with 5-LOX/COX inhibition or combinations of agents that represent different modes of action provide a practical approach for improving chemopreventive and therapeutic efficacy without unwanted side effects. For example, experiments were designed to test the chemopreventive/therapeutic efficacy of licofelone, a novel 5-LOX/COX-inhibitor. To test the efficacy of licofelone (50-300 ppm in diet) in the colon, we utilized azoxymethane (AOM)-induced rat colon carcinogenesis and Apc-min intestinal tumor model and AOM-induced rat colon adenocarcinoma model. In the rat colon model, licofelone suppressed AOM-induced colonic aberrant crypt foci (ACF) in a dose-dependent manner (Total ACF, $p<0.0.05$-0.0001; multicrypt foci, $p<0.01$–0.0001); similarly, Apc-min intestinal tumor formation was also significantly suppressed ($p<0.01$–0.0001). We will discuss on going progress on the combinational targets and identification select chemopreventive agents and promise for the human clinical trials.

419 Radiation as Immunotherapeutic

Andrew Raubitschek, David Colcher

City of Hope, Duarte, CA

Radiation is normally viewed as a directly cytocidal treatment modality causing double strand DNA breakage. However, sublethal irradiation can have profound consequences on the expression of surface proteins, which may then be recognized by the immune system leading to the subsequent destruction of the irradiated cells through immunologic mechanisms. We have been investigating the potential of utilizing this effect in immunotherapy, initially with radioimmunotherapy. Most animal models have utilized heterotransplanted tumors, making it impossible to evaluate the immunotherapeutic potential of RIT. We established a CEA transgenic mouse on a C57Bl6 background and CEA transfected MC38. We have been using the murine antibody, T84.66 ,which processes nanomolar affinity for CEA. When this antibody is conjugated with the DOTA, it can be labeled with both Indium-111 for imaging and Yttrium-90 for therapy. Doses of greater than 80uCi-Yttrium-90-labeled T84.66 will cure the majority of 10-day-old transplanted CEA-MC38 tumor-bearing mice. When the cured mice are challenged with fresh tumor, they are not immune, and tumors grow with the same kinetics as if the mouse had received no previous treatment. When tumor-bearing mice are treated with 60uCi of Yttrium-90-labeled T84.66, greater than 50% of the mice will be cured. Surprisingly, the cured mice were immune to rechallenge with either the CEA-MC38 or MC38. Evaluation of the cured mice demonstrated a significant increase in antigen-specific gamma interferon producing T cells. The numbers of antigen-specific T cells increased dramatically on rechallenge. We have been developing anti-CEA antibody-IL2 fusion proteins. These proteins show significant activity in the same animal model as above; however, they rarely cause complete tumor regression of 10-day-old CEA-MC38 tumors. We therefore combined RIT and IL2 fusion proteins. Animals with 10-day-old CEA-MC38 were treated with 40uCi of Yttrium-90-labeled T84.66, a dose that slows tumor growth without curing any animals. The RIT was followed 1 week later with three doses of anti-CEA-IL2 fusion protein administered on a MWF schedule. All the animals treated with the combination were cured of their tumors, and all of the animals were immune to tumor rechallenge. Mechanistic studies are underway to determine the role of effects on the tumor microenvironment and systemic immune system so that this synergy can be utilized clinically.

420 Blocking Tumor/Macrophages Cross-Talk With Anti-Mannose Receptor Recombinant Antibodies to Trigger Tumor Rejection

Nathalie Scholler[1], Denarda Dangaj[1,2], Raphael Sandaltzopoulos[2], George Coukos[1]

[1]Ovarian Cancer Research Center, Department of Obstetrics and Gynecology, School of Medicine, University of Pennsylvania; [2]Department of Molecular Biology and Genetics, University of Thrace, Greece

Macrophages are an essential constituent of the tumor microenvironment, and their presence has been correlated with increased angiogenesis and tumor growth. Tumor microenvironment polarizes macrophages toward a tumor-associated phenotype (TAM) or M2, that overexpresses mannose receptor (MR) and downregulates secreted levels of IL12. Independent works showed that MR cross-linking with anti-MR mAb causes DC differentiation into APC promoting T-cell anergy and suggested that macrophage phenotype switch is linked to a "chemical conversation" between tumor and macrophages through exchange of soluble mediators. Yet, the exact mechanisms underlying macrophage phenotype switch remain to be elucidated. To test whether soluble glycoproteins released by tumor cells could cause macrophage polarization toward M2 through binding to MR, we generated anti-MR recombinant antibodies (scFv) and assessed their function on macrophages in an in vitro cell model system. We established a model system of cell co-culture in transwell, allowing chemical exchanges between human monocyte-derived M1 or M2 macrophages and human ovarian cancer cell line OvCar3 that expresses mesothelin, a soluble glycoprotein. We first confirmed macrophage phenotype switch from M1 to M2 after 3 days of co-culture with OvCar3 cells. We then showed by flow cytometry analysis that tumor-released mesothelin could bind to macrophage cell surface and that the binding was greater on M2 than on M1 macrophages. Second, we generated a novel yeast-display recombinant antibodies (scFv) library from human origin that we screened with a yeast-expressed MR recombinant protein, and we identified three scFv against MR. We monitored the phenotype switch of M1 macrophages toward M2 during co-culture with tumor cell in the presence or in the absence of mannan, a ligand for MR, or of anti-MR scFv. By flow cytometry and qRT-PCR, we showed that all the novel anti-MR scFv could completely or partially prevent the binding of tumor-released mesothelin to macrophages, and we demonstrated that one anti-MR scFv could prevent macrophage polarization toward M2 during co-culture with tumor cells. Our findings indicate for the first time that mesothelin glycoprotein binds to MR and that tumor-induced M2 macrophage polarization can be blocked with anti-MR scFv. Our results also suggest that tumor-secreted glycoproteins contribute to M2 macrophage polarization through MR binding, which could be another strategy developed by tumors for immunomodulation. Finally, we propose that anti-MR scFv could prevent macrophage phenotype switch in vivo, allowing blocked or delayed tumor growth.

421 PAM4 Anti-Mucin Antibody for the Detection and Treatment of Pancreatic Cancer

Robert M. Sharkey[1], David V. Gold[1], Habibe Karacay[1], William A. Wegener[2], Edmund A. Rossi[3], William J. McBride[2], Chien-Hsing Chang[2], David M. Goldenberg[1]

[1]Center for Molecular Medicine and Immunology, Belleville, NJ; [2]Immunomedics, Inc., Morris Plains, NJ; [3]IBC Pharmaceuticals, Inc., Morris Plains, NJ

PAM4 binds to a unique epitope expressed specifically in pancreatic cancer (PC) mucin. The antigen is detectable in PC patients with a greater specificity and sensitivity than CA19-9, and immunohistology found the antigen in very early pre-cancerous lesions but absent in normal or non-malignant pancreas. 90Y-PAM4 IgG showed enhanced responses when combined with gemcitabine (GEM). The MTD for a single injection of 90Y-humanized PAM4 IgG was determined. A Phase Ib trial is examining a fractionated 90Y-PAM4 IgG treatment combined GEM (three weekly injections of 90Y-hPAM4 IgG, each followed 2 days later with 200 mg/m2 of GEM). Patients have tolerated a total cumulative dose of 36 mCi/m2 90Y-hPAM4 IgG (3 x 12 mCi/m2), and escalation is continuing. Several patients at earlier dose levels (i.e., cumulative dose of ~20 and 27 mCi/m2) received 2–3 additional treatment cycles after their first without dose-limiting toxicity. Anti-tumor responses have been reported, with one-half of ten assessable patients showing shrinkage or stabilization accompanied by a fall of CA19.9 in some, and with three patients having a partial response by RECIST criteria. A second-generation bispecific antibody (bsMAb) pretargeting system is now under development. BsMAb pretargeting targets radionuclides to tumors with a high signal, often rivaling the uptake of a directly radiolabeled IgG, but with much higher tumor/nontumor ratios, allowing for better therapeutic responses with less hematologic toxicity. Our system uses a novel tri-Fab, recombinant protein (TF10) that binds divalently to the tumor antigen and monovalently to a synthetic hapten, histamine-succinyl-glycine (HSG). Imaging studies in animal models showed exceptional tumor uptake (~20–25% injected dose per gram) and high tumor/nontumor ratios within 3 h of the HSG-peptide's injection. Pretargeting is highly sensitive, capable of detecting micrometastatic tumors as small as 0.3 mm in diameter not detected with 18F-FDG. Pretargeting is also more specific, being better able to distinguish tumor from inflammation than 18F-FDG. For therapy, a 90Y-labeled HSG-peptide has provided excellent anti-tumor responses alone that are enhanced when combined with GEM. Fractionated treatments were more effective than a single dose. In conclusion, the PAM4 antibody has shown promise as an in vitro immunoassay and immunohistochemical method for early and specific pancreatic cancer detection, as a directly-labeled radioimmunotherapeutic in clinical studies, and now in a pretargeting setting with a human pancreatic cancer xenografts model, showing improved efficacy in combination with GEM.

422 Targeting CD40 for Immunotherapy of Cancer

Robert H. Vonderheide

Abramson Cancer Center, University of Pennsylvania School of Medicine, Philadelphia, PA

The cell-surface molecule CD40 is a member of the tumor necrosis factor receptor superfamily and is broadly expressed by immune, hematopoietic, vascular, epithelial cells, and certain tumor cells. CD40 itself lacks intrinsic signal-transduction activity but rather mediates its effects via an intricate series of downstream adapter molecules that differentially alter gene expression depending on cell type and microenvironment. CD40 is best appreciated as a critical regulator of cellular and humoral immunity via its expression on B lymphocytes, dendritic cells, and monocytes. CD40-ligand, also known as CD154, is the primary ligand described for CD40 and is expressed by activated T cells. Signaling via CD40 activates antigen presenting cells (APC) both in vitro and in vivo. Physiologically, this signal represents a major component of T cell help and mediates in large part the capacity of helper T cells to "license" APC. Although resting APC may drive T cell tolerance and anergy, fully activated or "licensed" APC autonomously orchestrate effective and productive T cell responses.

To test the hypothesis that CD40 activation can induce clinically meaningful anti-tumor cellular immunity in patients, we have evaluated the novel agonist CD40 monoclonal antibody (mAb) CP-870,893 (Pfizer) for safety, immune pharmacodynamics, and efficacy in patients with advanced cancer. Two phase I studies have been completed in collaboration with Pfizer Corporation and colleagues at the Abramson Cancer Center, Moffitt Cancer Center, Sara Cannon Cancer Center, South Texas Accelerated Research Therapeutics, and the Angeles Clinic. These are a single-infusion study of CP-870,893 and a weekly repeated dose study. The chief side effect observed has been transient and manageable cytokine release syndrome. Immunologically, activation of antigen-presenting cells has been observed, in some cases with evidence of induction of anti-tumor T cell responses. In the single-infusion study, 4 out of 29 patients, all with metastatic melanoma, had partial responses. In the weekly study, no objective responses were observed, and the interval of dosing has been increased for subsequent studies. In line with multiple preclinical models, we are currently investigating the combination of CP-870,893 with chemotherapy in phase I studies. Other combinations such as with vaccines or anti-CTLA-4 mAb also warrant further investigation.

423 Determinants of Tumor Sensitivity to EGFR-Targeted Antibodies

Louis M. Weiner[1], Sandra A. Jablonski[1], Yong Tang[1], Ilya Serebriiskii[2], Igor Astsaturov[2], Erica Golemis[2]

[1]Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC; [2]Fox Chase Cancer Center, Philadelphia, PA

This project has focused on understanding the critical structural determinants of anti-tumor antibodies that promote tumor-specific, antibody-dependent cellular cytotoxicity (ADCC), defining the antibody structural features and treatment strategies that maximize ADCC and promote the induction of adaptive immunity. Recently, our focus has shifted to analyzing the tumor-intrinsic factors that sensitize tumor cells to antibody therapy. We hypothesized that siRNA screening focused on genes functionally linked to the EGFR signaling pathway would identify tumor-intrinsic genes that regulate the tumor cell response to antigen engagement and ADCC promotion by monoclonal antibodies. To test this hypothesis, we have developed and applied a customized 638-element siRNA library containing genes known to functionally interact with EGFR (the EGFR functional "interactome"). Using this library, we identified a restricted number of genes whose knockdown selectively alters tumor cell viability in the presence of panitumumab and other EGFR inhibitors. We have identified "clusters" of genes known to act together in discrete subpathways, which we predict will be important for regulation of EGFR-family-directed signaling inhibition and ADCC. This project will dissect immunologic and signaling mechanism contributions to antibody efficacy and then identify efficacy-sensitizing genes for signaling and ADCC. Specific Aim 1 is to determine the roles of signaling inhibition and ADCC in mediating the efficacy of EGFR pathway-directed monoclonal antibodies. Specific Aim 2 is to define the elements of the EGFR interactome that modify target cell death in response to antibody engagement using cell culture-based functional tests to confirm the mechanisms by which siRNA depletion enhances tumor cell killing and in vivo models. In vitro and in vivo validation studies will identify new antibody-based therapy combinations. These studies will also identify those genes that regulate cellular sensitivity to EGFR inhibition and ADCC sensitivity. Specific Aim 3 is to determine the influence of mutations in critical signaling genes on EGFR antibody-targeted cytotoxicity. The completion of these aims will yield an improved understanding of the critical mechanisms that underlie successful antibody therapy and will form the basis for future monoclonal antibody treatment strategies.

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424 Successful Immunotherapy With IL-2/anti-CD40 Coincides With Specific Chemokine-Mediated Mitigation of an Immunosuppressive Tumor Microenvironment for the Improved Treatment of Renal Cell Carcinoma

Jonathan M. Weiss[1], Jeff Subleski[1], Tim Back[1], Veronica Hall[1], Tony Scarzello[1], Jim Stauffer[1], Tim Chan[1], Kory Alderson[2], William J. Murphy[2], Robert H. Wiltrout[1]

[1]Cancer and Inflammation Program, Laboratory of Experimental Immunology, National Cancer Institute, Frederick, MD; [2]Department of Dermatology, University of California, Davis

Successful immunotherapy against tumors including renal cell carcinoma (RCC) requires coordinated engagement of the immune system. Interleukin (IL)-2 is an immune-stimulating cytokine that promotes anti-tumor responses in preclinical RCC models and the clinic. Similarly, initial findings have shown objective clinical responses resulting from the treatment of patients with established solid tumors with agonistic αCD40 antibody. As single agents, both IL-2 and αCD40 mediate only partial, transient antitumor effects; however, their combined use may lead to substantially more effective anti-tumor responses. Similar to IL-2/IL-12 currently used in the clinic, IL-2/αCD40 combination therapy resulted in synergistic anti-tumor responses. These effects were dependent upon interferon gamma (IFNγ) and the CD40-mediated induction of IL-12 expression. The anti-tumor effect achieved by αCD40 alone was qualitatively different from that mediated by IL-2/αCD40 treatment. Whereas αCD40-mediated leukocyte recruitment into tumors was dependent on CCR2- and monocyte chemoattractant protein-1 (MCP-1), the effective anti-tumor therapy using IL-2/αCD40 was independent of CCR2. Furthermore, IL-2/αCD40 therapy achieved the augmentation of effector CD8+ T cell recruitment by IFNγ-induced chemokines concomitant with removal of CD4+/FoxP3+ regulatory T cells, myeloid-derived suppressor cells, and reduced Th2 chemokine expression within the tumor microenvironment that were not observed with αCD40 alone. Interestingly, IL-2/αCD40 induced an increase in peripheral Tregs that did not prevent the anti-tumor response, suggesting that only the redistribution of effector and regulatory leukocytes within the tumor microenvironment was predictive of therapeutic success. Both IL-2 and αCD40 are being used in cancer patients as monotherapy; however, objective responses have been observed in only a minority of patients. Our data suggest that the therapeutic efficacy of αCD40 may be limited by its dependency upon MCP-1 and inability to remove Tregs and MDSC within the tumor microenvironment, allowing for eventual tumor progression. In contrast, the synergistic combination of αCD40 with IL-2 not only potentiated the antitumor effect of either treatment, but also elicited the expression of Th1 chemokines associated with favorable prognosis in RCC, augmented effector leukocytes and concomitant removal of suppressive cells specifically within the tumor microenvironment. Thus, combined IL-2/αCD40 treatment holds substantial promise for clinical cancer treatment.

425 Alpha Particle Immunotherapy of Cancer

David A. Scheinberg, Michael R. McDevitt, Joseph Jurcic, Todd Rosenblat, Carlos Villa

Memorial Sloan-Kettering Cancer Center

PO1-CA33049, Project 1: The long-term goal of this project, in its 15th year, is to develop selective, highly potent therapies by targeting antigens on cancer cells using novel antibody-based agents. We have successfully developed native, alpha-, and beta-emitting radiolabeled mAb constructs and taken them to phase I, II, and III trials. We have completed a series of studies with the HuM195 mAb reactive with CD33 in acute leukemia and promyelocytic leukemia, reaching phase II trials under NIH grant sponsorship. In addition, development of alpha- and beta-emitting constructs has led to a series of phase I and phase II trials. Preclinical development of the first targeted atomic alpha nanogenerator was completed, allowing human trials to begin. The preliminary success of our targetable in vivo alpha generator (Ac-225 nanogenerator) in patients with AML led us to ask whether more sophisticated and effective targeted nanodevices could be constructed for injection. Limitations exist with the current constructs: (1) the number of therapeutic atoms delivered to the cells, even in accessible leukemias is quite small (1 in 500 mAb carries an Ac-225 using our optimized chemistry); (2) hence, in its current form, the approach may be limited to radiosensitive cancers of the blood and marrow or to the tumor neovasculature rather than to more inaccessible solid tumors; (3) there is no reliable way to determine the dose of drug to the target or off-target organs in order to predict best doses or anticipate toxicity, because the alpha-emitting atom cannot be imaged at the tiny doses used. Altering these features would provide a vast improvement for an agent with potential for high non-target toxicity and would allow RIT of solid tumors. One promising nanomaterial is the single-walled carbon nanotube, SWNT. Although pristine as-produced carbon nanotubes are highly inert, insoluble in aqueous media, and potentially toxic, soluble functionalized nanotubes appear to be safe. In addition, the small diameter of SWNT affords them unique biological properties, particularly with respect to their pharmacokinetics and cellular interactions. Furthermore, because of nanotubes' large available surface area, they could potentially be radiolabeled to highly specific activities, enabling advances in specific activity of therapeutic constructs for amplification of radioisotope delivery as well conjugation of multiple ligands to confer multifunctionality such as imaging and therapy simultaneously.

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